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Ikunosuke OKADA, Professor, Dr.

Hiroshi NIINO, Professor, Dr.

Itsuo KUBO, Professor, Dr.

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STUDIES ON KAMABOKO-FORMING ABILITY OF MYOSIN

(Part II)*

Hiroshi HIRANO

(Received November 1, 1952)

III. PROPERTIES OF EUMYOSIN

III, 1. Introduction

The author has previously pointed out that *Kamaboko*-forming constituent of fish flesh is myosin (HIRANO, 1942 d). He has those times discovered that the *Kamaboko*-forming ability is ascribable to eumyosin, a main component of classical myosin, named as such by him,[21] and recently published a procedure by which eumyosin can be isolated with ease (HIRANO, 1951).

Classical myosin is thus thought to contain a second non-*Kamaboko*-forming component being named by the author pseudomyosin. In the routine procedure of *Kamaboko*-manufacturing, these two protein fractions, eumyosin and pseudomyosin, are presumed to behave as a sort of complex to form *Kamaboko*-jelly, because neither of them can be brought into solution by a single extraction without contamination with the other.

In order to comprehend the mechanism of *Kamaboko*-formation phenomenon, however, it seems to be far more advisable to treat the properties of isolated eumyosin than to treat those of classical myosin, for the former is the unique *Kamaboko*-forming entity. The author intends in this chapter to report the results of observation on several properties of eumyosin, which seem to be available in drawing an inference about the problem, and also in making some considerations concerning the principles underlying these properties.

III, 2. Behaviors in Electrolyte-Solutions

Eumyosin, being an essential component of classical myosin which should have in itself *Kamaboko*-forming ability, is expected to behave in neutral salt solutions closely like classical myosin, *e.g.*, to be subject to remarkable salting-in, giving rise to a viscous sol. While the behaviors of eumyosin in neutral salt solutions seem to be fairly specific like those of classical myosin, the observation on the behaviors in aqueous solutions of simple acid or base such as HCl or NaOH respectively may presumably contribute greatly to comprehending

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* Among numerous errors having appeared in the previous paper, Part I (HIRANO, 1951), most serious ones are pointed out as follows:

Place	*Erroneous*	*Correct*
Page 219, Table 3, Column 4	of bleached fish	of fish before bleaching
Page 219, Line 12	16.00%	16.71%
Page 239, Line 18	Table 2	Table 7

21) *Cf.* foot note 8) in HIRANO, 1942 d.

Table 10. Behaviors of Eumyosin a: Samples prepared

I Number of Sample	II Appearance of sample	III Filtration velocity	IV Appearance of filtrate
1	Uniformly semitransparent elastic liquid.	Very small	Clear
2	Ditto	Ditto	Ditto
3	Ditto	Ditto	Ditto
4	Ditto	Ditto	Ditto
5	Uniformly slightly turbid.	Ditto	Ditto
6	Very small amount of clear supernatant was separated.	Small	Ditto
7	Moderate amount of clear supernatant was separated.	Moderate	Ditto
8	Large amount of clear supernatant was separated.	Very large	Ditto
9	Moderate amount of clear supernatant was separated.	Large	Ditto
10	Small amount of clear supernatant was separated.	Moderate	Ditto
11	Uniformly slightly turbid.	Small	Ditto
12	Uniformly hazy.	Very small	Ditto
13	Uniformly almost clear.	Large	Hazy
14	Ditto	Very large	Ditto
15	Ditto	Ditto	Ditto
16	Ditto	Ditto	Ditto

* The operation to isolate eumyosin was completed on Oct. 21, 1941.
Columns VI and VII: It is inferred that the sampling of the starting suspension
Column VIII: Viscosity-measurements were carried out at 25°C.

the ionic structure of eumyosin since most proteins behave rather in common in such solutions.

III, 2, 1. Solubility and Viscosity in HCl- or NaOH-Solutions

Experimental. An isoelectric precipitate which had been prepared by acidifying the routine preparation of eumyosin (*cf.* HIRANO, 1951) with HCl to pH 5.5 was used in this experiment.

Every 100 *cc* of the homogenized suspension obtained by stirring up the isoelectric precipitate were added with varying amounts of 0.1 *N* HCl, 0.1 *N* NaOH, or *N* NaOH, while the total volume of each sample was adjusted to a definite value (200 *cc*) by the addition of distilled water, so that a series of samples were obtained, which were constant in total concentration of the protein but variant in pH. After the appearance of each sample was scrutinized, pH value, solubility,[22] and the relative viscosity (after filtration) at 25°C were estimated

22) In this paper, the solubility of eumyosin is expressed very often in the percentage of the nitrogen found in liquid phase on the basis of the total nitrogen in the sample. Such a value, however, is of quite a different meaning from the normally defined solubility and seems rather to represent "peptizability," since classical myosin has already been accepted to exist in a state of micelle (*cf.* ARDENNE and WEBER, 1941). In the following descriptions, however, the author shall generally continue to use "solubility" for the value despite its discordance with the normal definition as required in phase rule.

in HCl or NaOH Solutions.*
onOct. 27, 1941.

V pH (18°C)	VI Filtered N in *per cent* of total N	VII Protein in *gram* per 100 *cc* of filtrate (C_p)	VIII Relative viscosity (η_R)	IX Intrinsic viscosity $\left(\frac{log\,\eta_R}{C_p}\right)$
2.147	20.3	0.0359	1.148	1.670
2.45	17.8	0.0315	1.135	1.745
2.80	9.49	0.0168	1.060	1.505
3.15	3.85	0.0068	1.021	1.324
3.55	2.96	0.0052	1.015	1.23
4.00	2.17	0.0039	1.007	1.00
4.50	1.29	0.0023	1.002	0.38
5.80	0.59	0.0011	1.000	0.00
6.95	1.18	0.0021	1.003	0.62
7.15	1.29	0.0023	1.004	0.76
7.20	2.07	0.0037	1.008	0.94
7.35	5.14	0.0091	1.020	0.95
10.50	100.4	0.1778	1.468	0.936
11.45	101.2	0.1790	1.300	0.618
12.10	100.4	0.1778	1.192	0.387
12.45	99.8	0.1782	1.114	0.262

had not uniformly been accomplished throughout all the samples.
Column IX: *Cf.* foot note 24.

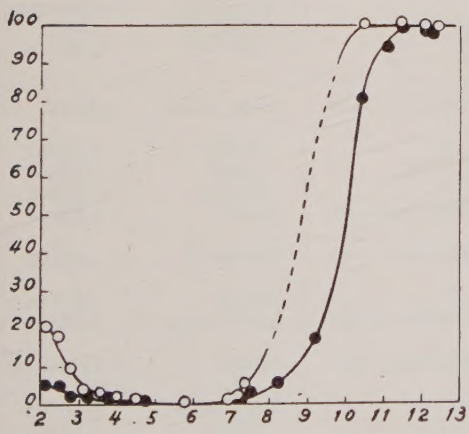


Fig. 4. Solubility of eumyosin in HCl or NaOH solutions. Abscissa: pH of HCl or NaOH solutions containing eumyosin. Ordinate: Filterably dissolved N in percent of total N. Open circles: Series A (rather fresh preparation). Solid circles: Series B (rather aged preparation).

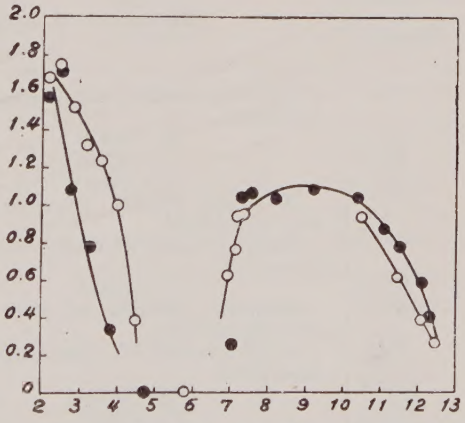


Fig. 5. Intrinsic viscosity of eumyosin dissolved in HCl or NaOH solutions at 25°C. Abscissa: pH of HCl or NaOH solutions containing eumyosin. Ordinate: Intrinsic viscosity. Open circles: Series A (rather fresh preparation). Solid circles: Series B (rather aged preparation).

for each sample. The results of experiment obtained with eumyosin preparations of the same origin but of different ages are given in Tables 10 a and 10 b.

One of the most remarkable affairs experienced in this experiment was that pH's of the samples were driven gradually towards the isoionic point with the advance of time after the addition of HCl or NaOH to the suspensions; at the same time, falls in viscosity were observed especially in evidence in basic samples, pointing to that eumyosin particles underwent gradual dissociation into smaller units so as to develope a larger amount of ionic groups which enter the interaction with the acid or base added to the system. Each sample, therefore, was filtered after a virtual equilibrium had been attained. Nevertheless, the experimental results of the samples prepared from aged eumyosin (Table 10 b) do not agree so well with those of the samples from rather fresh eumyosin (Table 10 a). The same situations are visible at once in Figures 4 and 5.

Considerations on the results. As it is seen in Tables 10 a and 10 b as well as in Figure 4, eumyosin is practically insoluble in a pH range slightly acid to pH 7, while it dissolves more or less appreciably in HCl or NaOH solutions on the outside of the said pH range. Sols of eumyosin, not only having extremely high values of intrinsic viscosity but also behaving as prominently flow-birefringent, may well be expected to contain huge fibrous particles like those of classical myosin.

The apparent dispersion of such particles into an aqueous solution is considered to be realized in two ways: (1) When the hydration of these particles are extremely promoted so that water is imbibed wholly throughout the pile of

Table 10. b: Samples

I Number of Sample	II Appearence of Sample	III Filtration velocity	IV Appearence of filtrate
1	Small amount of clear supernatant was separated.	Very small	Clear
2	Ditto	Ditto	Ditto
3	Ditto	Ditto	Ditto
4	Ditto	Ditto	Ditto
5	Moderate amount of clear supernatant was separated.	Small	Ditto
6	Large amount of clear supernatant was separated.	Moderate	Ditto
7	Ditto	Large	Ditto
8	Moderate amount of clear supernatant was separated.	Very large	Ditto
9	Small amount of clear supernatant was separated.	Small	Trace of haze
10	Ditto	Very small	Ditto
11	Ditto	Ditto	Ditto
12	Very small amount of clear supernatant separated.	Small	Hazy
13	Trace of the separation of clear supernatant.	Moderate	Ditto
14	Uniformly filled with voluminous colonies.	Large	Ditto
15	Uniformly hazy liquid.	Ditto	Ditto
16	Uniformly slightly hazy liquid.	Very large	Ditto

the swollen-up particles, a sort of fluid system being accompanied by thixotropy may possibly be yielded in so far as little attractive forces are prevailing between them. (2) If the particles are disintegrated or dissociated into sufficiently smaller units such as accessible to the disgregating thermal motion of the surrounding water molecules, a stable sol may be given rise to. In pH ranges where extraordinary high-viscosities are exhibited, eumyosin particles are presumed to be dispersed by the way (1) into a sort of thixotropic jelly with an extremely low consistency. On the contrary, in the pH range where eumyosin is able to be dissolved into a fairly concentrated but not viscous solution, *e.g.*, in the range alkaline to pH 11, it is supposed that the disintegration of particles, whether reversible or irreversible, takes place as expected in the way (2).

On the other hand, it is conceivable that the disintegration of eumyosin in NaOH solutions proceeds under certain circumstances *reversibly* since the author has successfully prepared the protein by extracting bleached fish with an ice-cold solution of NaOH at a pH of about 12. Indeed, as will be described later (*cf.* III, 4.), eumyosin is not deprived of its *Kamaboko*-forming ability by mere being dissolved in an NaOH-solution unless its intrinsic viscosity drops to become lower than about 0.2 still corresponding to as large a value as nearly two times the viscosity of the solvent.

Being based on these considerations, the extraordinarily high values of intrinsic viscosity found in HCl solutions or in slightly alkaline NaOH solutions lead to the assumption that eumyosin in these solutions exists forming huge micelles. Furthermore, the fact that these solutions are always but of low pro-

prepared on Nov. 17, 1941.

V pH (18°C)	VI Filtered N in *per cent* of total N	VII Protein in *gram* per 100 *cc* of filtrate (C_p)	VIII Relative viscosity (η_R)	IX Intrinsic viscosity $\left(\frac{log\,\eta_R}{C_p}\right)$
2.150	5.02	0.0090	1.033	1.57
2.50	4.87	0.0087	1.035	1.71
2.78	2.24	0.0040	1.010	1.08
3.24	1.86	0.0033	1.006	0.78
3.80	1.46	0.0026	1.002	0.33
4.74	0.88	0.0016	1.000	0.00
7.05	0.97	0.0017	1.001	0.25
7.30	1.17	0.0021	1.005	1.04
7.57	2.95	0.0053	1.013	1.06
8.25	5.36	0.0096	1.023	1.03
9.22	17.2	0.0308	1.080	1.08
10.42	80.6	0.1444	1.413	1.04
11.10	94.3	0.1690	1.402	0.87
11.50	99.7	0.1804	1.382	0.78
12.10	98.6	0.1747	1.262	0.58
12.30	98.0	0.1765	1.176	0.40

tein concentrations despite their remarkable slow rates at which they pass through filter paper implies that eumyosin micelles may certainly be extremely asymmetric in their configurations so as swept large volumes when they move rotatorily.

In rather strongly acid or basic solutions of eumyosin, in which further formation of cationic or anionic groups may be possible within the individual molecules, original anionic or cationic groups respectively may be disionized more or less substantially if not entirely, leaving the corresponding positive or negative net charge on the molecules; and the repulsive interaction between the net charges pertaining to the adjacent molecules within the same micelle may well lead to the disruption of the micelle, thus effecting the decrease in viscosity of the solutions. The dissolution tendency of eumyosin, therefore, may be promoted by the increase in net charge realized on the intramicellar molecules.

If eumyosin micelles were formed only by salt-linkages, it would have been expected that the micelles were formed most easily in the pH range where net charge becomes minimal without appreciable diminution in total charge, namely, in the pH range around the isoelectric point where eumyosin becomes least soluble. The intrinsic viscosities found in such a pH zone, however, are not very large as seen in Tables 10 a and 10 b, and also in Figure 5; but any conclusive discussion such as this cannot positively be made on the present data since their accuracies are extremely poor in the isoelectric zone due to the practical insolubility of eumyosin. As presumed from Figure 4 and 5, the isoelectric precipitate of eumyosin used in this experiment became less and less soluble during the storage, suggesting that the eumyosin particles are always subject to some concreting forces which grow slowly stronger and stronger over the salt-linkages which are expected in turn to be always reversible in nature.

From such reasoning, it follows that some factors other than intermolecular salt-linkages should also be introduced to interprete the motive for the micelle formation; but the problem will be reserved for a later discussion (*cf.* IV, 2, 2).

Now as it will be seen from the sedimentation data in III, 2, 7 as well as from one of the curves in Figure 15 (III, 3, 1, 3), both of which concern preparations freshly precipitated and hence may be looked to have not yet undergone coagulation, the solubility curve of eumyosin often does not run symmetrically against pH around the isoelectric point, *i.e.*, the rate of increase in solubility is far more hesitating in the basic range than in the acid one to the isoelectric point.* This implies that, while a main portion of ionizable groups contained in an eumyosin molecule consists of rather weakly-ionizing anionic groups as well as of rather strongly-ionizing cationic groups, not a small portion of them is composed of phenolic-hydroxyl and imidazolium groups, respectively, whose dissociation constants are both supposed to be situated in the neutral pH zone even when they belong to structural units of eumyosin molecules.

Finally, the fact that the solubility in strong HCl solutions appears somewhat lower than that in strong NaOH solutions, being more conspicuous with the case of rather aged eumyosin deposit, may be due not only to the numerical preponderance of anionic groups over cationic groups within the individual mole-

* *Cf.* also the swelling curve of press-residue of muscle in WEBER's paper (1925 b).

cules but to the essential difference in hydration degree* as well as in configuration between these groups. When an eumyosin deposit is peptized into a sol by the action of HCl or NaOH, these agents are expected to function as wedges which facilitate the eumyosin micelles constituting the deposit to be dissociated into sufficiently fine units which can be dispersed through thermal agitation. The more deeply the wedges are driven into the micelles, the more readily will be the micelles dissociated. The wedges mentioned above are not but the net charge with which the eumyosin molecules constituting the micelles are provided by the said agents. Whereas the positive net charge acquired by eumyosin in acid solutions consists predominantly of δ-guanidinium groups of arginyl residues and ε-ammonium groups of lysyl residues which are disposed a good distance off from the backbone of the molecule, the negative net charge established in basic solutions consists predominantly of β-carboxyl groups of asparagyl residues and γ-carboxyl groups of glutamyl residues which are situated closely to the molecular backbone . It may well be expected therefore that basic solutions give rise to repulsive forces acting on the intramicellar molecules more effectively than acid solutions do, facilitating the micelles the more effectively to be dissociated into simpler particles so that the higher solubility is exhibited.

III, 2, 2. Isoionic Point

It may well be expected that, from the results given in III, 2, 1, the isoionic point of eumyosin is situated between pH 5 and pH 6. It was verified by an electrophoretic experiment that eumyosin behaves isoelectrically in the said pH zone as follows :

A set of saturating bodies at various pH's were prepared by the differen-

Table 11. Electrophoresis of Eumyosin-precipitate at Various pH's.

Number of sample	pH	Appearance of saturating body	Date of observation (1941)	Direction of electrophoresis	Presumed sign of net charge on the precipitate	Remarks
1	9	Greenish blue, thixotropic jelly.	Oct. 22	towards Anode	—	Migrated at 6 *mm* per hour.
2	8	Ditto	Oct. 22	Ditto	—	
3	7	Ditto	Oct. 25	Ditto		
4	6.0	Colorless, semi-transparent, voluminous, non-thixotropic.	Oct. 23	Ditto	—	Migrated at 5 *mm* per hour.
5	5.5	White, almost opaque, voluminous, non-thixotropic.	Oct. 26	?	±	Stood still for three hours.
6	5.0	Ditto; but more voluminous.	Oct. 23	towards Cathode	+	Migrated at 0.6 *mm* per hour.

* From the data of the apparent molal volume of amino acids in watery medium, which was measured by SHIMURA (1949), it is inferred that they become more strongly depressed in hydration when made into cation than into anion. Also with the case of proteins, their carboxyl groups would become hydrated on ionization more heavily compared with their ammonium groups.

tial addition of 0.1 *N* HCl to a concentrated sol of eumyosin having been dispersed in a dilute solution of NaOH. The migration of the precipitates was observed in a PAULI transportation apparatus under the application of DC 110 volts generated from an induction motor. The results are given in Table 11.

From these results, it is obvious that the isoelectric point, presumably also the isoionic point, of eumyosin lies near pH 5.5, deviating slightly towards a basic reaction but almost in accordance with the critical data for rabbit myosin reported by HOLLWEDE and WEBER (1938). It is also understood that eumyosin is not isoelectric at pH 8.5, though at this pH eumyosin is thrown down violently out of solution forming astonishingly coarse agglomerates. (The formation of the characteristic agglomerates at pH 8.5 will be discussed later (*cf.* IV, 3.)).

III, 2, 3. Combination with Acid or Base

Like other proteins, eumyosin behaves as a cation in an aqueous solution whose reaction is acid to the isoionic point, combining H^+ ion, while it behaves as an anion in such that is basic to the isoionic reaction, releasing H^+ ion, or

Table 12. H^+- or OH^--ion Fixed by Eumyosin
(Total volume of samples = 200 *cc*; Average concentration of eu-

Number of sample		H^+ or OH^- added to isoionic eumyosin (*millimol*)	pH (18°C)	Indicator used for pH– determination	Activity of H^+ or $OH^- \times 10^3$	
Series A,	1	H^+ 3.19	2.147	(Electrometric)	a_H	7.13
	2	1.59	2.45	Methyl orange		3.55
	3	0.79	2.80	〃		1.58
	4	0.39	3.15	〃		0.71
	5	0.19	3.55	〃		0.28
	6	0.09	4.00	〃		0.10
	7	0.04	4.50	Methyl red		0.03
	8	OH^- 0.01	5.80	〃	a_{OH}	0.00
	9	0.06	6.95	Bromthymol blue		0.00
	10	0.11	7.15	〃		0.00
	11	0.24	7.20	〃		0.00
	12	0.41	7.35	〃		0.00
	13	0.81	10.50	Salicyl yellow		0.19
	14	1.61	11.45	〃		1.70
	15	3.21	12.10	Tropäolin O		7.59
	16	6.41	12.45	〃		17.0
Series B,	1	H^+ 3.14	2.150	(Electrometric)	a_H	7.08
	2	1.54	2.50	Methyl orange		3.16
	3	0.74	2.78	〃		1.66
	4	0.34	3.24	〃		0.72
	5	0.14	3.80	〃		0.16
	6	0.04	4.74	Methyl red		0.02
	7	OH^- 0.06	7.05	Bromthymol blue	a_{OH}	0.00
	8	0.16	7.30	〃		0.00
	9	0.26	7.57	〃		0.00
	10	0.46	8.25	Phenolphthalein		0.00
	11	0.66	9.22	〃		0.01
	12	0.86	10.42	Salicyl yellow		0.15
	13	1.26	11.10	〃		0.72
	14	1.66	11.50	〃		1.91
	15	3.26	12.10	Tropäolin O		7.59
	16	4.86	12.30	〃		12.1

combining OH^- ion.

From the relation found between the amounts of HCl or NaOH added and the pH attained in the samples (before filtration) referred to III, 2, 1, the equivalent weight of eumyosin as a base or an acid, *i. e.*, the reciprocal value of the equivalent number of H^+ ion combined or released per unit gram of anhydrous eumyosin, is estimated as 400 or 300, respectively. These estimations are apparently not only far much smaller but also opposite in order by magnitude compared with the findings by SALTER (1926) on ox myosin; and they suggest that a remarkable portion of the side-chains of eumyosin molecule, even though not so large a portion as with pseudomyosin, may be composed of ionogenic groups as might be expected from the physiological function of myosin.

The data from which the above estimations have been derived are listed in Table 12. There, as the values of γ_H and γ_{OH}, activity coefficients of H^+ ion and OH^- ion respectively, those obtained by interpolation between the electrometrically-found values on dilute HCl and NaOH solutions at known concentrations have been used, neglecting the effect of the protein coexistence, while those of not very strongly acid or basic samples have been assumed to be equal

in HCl- or NaOH Solution, respectively.
myosin in samples=Series A: 1.78 *mg/cc*; Series B: 1.80 *mg/cc*).

Activity coefficient of H^+ or OH^-		Amount of H^+ or OH^- per unit *cc* of sample (*millimol* $\times 10^3$)						H^+ or OH^- fixed per unit *gram* of eumyosin (*gramequivalent* $\times 10^5$)	
		Added		Free		Fixed			
γ_H	0.62	H^+	15.95	H^+	11.50	H^+	4.45	H^+	250
	0.95		7.95		3.74		4.21		246
	1		3.95		1.58		2.37		133
	1		1.95		0.71		1.24		70
	1		0.95		0.28		0.67		38
	1		0.45		0.10		0.35		20
	1		0.20		0.03		0.17		10
γ_{OH}	1	OH^-	0.05	OH^-	0.00	OH^-	0.05	OH^-	3
	1		0.30		0.00		0.30		17
	1		0.55		0.00		0.55		31
	1		1.20		0.00		1.20		67
	1		2.05		0.00		2.05		115
	1		4.05		0.19		3.86		217
	0.78		8.05		2.18		5.87		329
	0.72		16.05		10.55		5.50		308
	0.64		32.05		26.6		5.45		306
γ_H	0.63	H^+	15.70	H^+	11.23	H^+	4.47	H^+	248
	0.98		7.70		3.22		4.48		249
	1		3.70		1.66		2.04		113
	1		1.70		0.72		0.98		55
	1		0.70		0.16		0.54		30
	1		0.20		0.02		0.18		10
γ_{OH}	1	OH^-	0.30	OH^-	0.00	OH^-	0.30	OH^-	17
	1		0.80		0.00		0.80		45
	1		1.30		0.00		1.30		72
	1		2.30		0.00		2.30		128
	1		3.30		0.01		3.29		183
	1		4.30		0.15		4.15		230
	0.83		6.30		0.87		5.43		302
	0.78		8.30		2.45		5.85		325
	0.69		16.30		11.00		5.30		294
	0.65		24.30		18.6		5.70		320

to unity. As the hydrolytic constant of water, K_W, BJERRUM and UNMACK's (1929) value, 0.61×10^{-14} at 18°C, has been adopted.

Remarks 1. pH values of samples No. 8, No. 9, and No. 10 of series B in Table 12 were found to be respectively 8.28, 8.68, and 8.81 immediately after the preparation;[23] they had dropped, however, down to such lower values as are given in the table, *i.e*, 7.30, 7.57, and 8.25 respectively, while such a gradual sort of pH-drift in process of time did not occur practically in the other samples.

A pH-drift of this kind is observable in general for eumyosin precipitates kept long in water when they are treated with a dilute solution of NaOH so as to become faintly basic in reaction. Though the pH-drift is considered to be caused by the peptization of precipitate, the rate of drift would be too small in HCl solutions and too large in strong NaOH solutions to be followed. It may also be presumed from such a viewpoint that ionic groups which are buried in the depth within the colonies constituting a precipitate attain only difficultly to ionization equilibrium. The marked discrepancy between the relations showing the acid- or base- combining capacities at various pH's in series A and B, which are illustrated in Figure 6, is found in the pH range (around 7–9) where the critical reaction for building-up or breaking-down of the characteristic large colonies of eumyosin is situated. Thus, the discrepancy implies that the micellar structure becomes spontaneously more and more compact while eumyosin is kept precipitated at the isoelectric point.

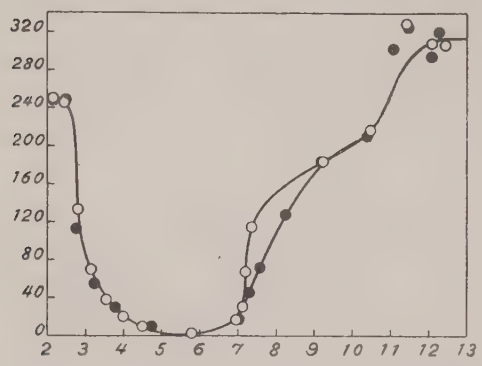


Fig. 6. Amount of H^+ or OH^- ion combined with eumyosin in HCl or NaOH solutions, respectively. Abscissa: pH of HCl or NaOH solutions containing eumyosin. Ordinate: H^+ or OH^- ion in gramequivalent $\times 10^5$. Open circles: Series A (rather fresh preparation). Solid circles: Series B (rather aged preparation).

III, 2, 4. Solubility in NaCl Solution

Eumyosin, being looked upon as a chief constituent of classical myosin, is expected to display profound salting-in when it is treated with an NaCl solution of moderate concentration. This expectation has been examined as follows:

Experimental. Every 100 *cc* of an eumyosin suspension having been freshly prepared by the routine procedure were added with 10 *cc* of 1.00 *M* potassium phosphate buffer at pH 7.0 (COHN, 1927), and further with calculated amounts of 5 *M* NaCl and of distilled water so that a series of samples in duplicate were obtained, each member of which had a definite value in total volume, 200 *cc*, and also in pH, 7.0, but was varied in NaCl content.

All the samples were stirred up from time to time and finally allowed to stand over a night under room temperature (the maximum was recorded at 18°

23) The corresponding initial values of pH, unfortunately, have not been recorded for series A.

C) so that virtual equilibrium might be attained. Next day, these samples, putting aside the controls, were filtered avoiding disturbance, and the amounts of dissolved nitrogen were determined for aliquot portions (50 *cc* were used in

Table 13. Solubility of Eumyosin in NaCl-Solutions at pH 7.

Number of sample	NaCl concentration	Appearance of sample	Solubility of eumyosin represented by			
			filterably dissolved N		non-centrifugibly dispersed N	
			per 100 *cc* of filtrate	in per cent of total N	per 100 *cc* of supernatant	in per cent of total N
	M		*mg*	%	*mg*	%
1	0	Clear mother liquor with volumious precipitate.	0.64	3.8	0.54	3.2
2	0.0625	Hazy mother liquor with rather compact precipitate.	2.88	17.3	3.52	21.2
3	0.125	Uniformly opalescent liquid with faint turbidity.	3.54	21.3	5.96	35.8
4	0.25	Ditto; but less turbid.	5.86	35.2	11.38	68.2
5	0.5	Almost transparently opalecent liquid.	6.34	38.1	15.12	90.6
6	1.0	Ditto; but less transparent.	7.72	46.5	16.60	99.5
7	1.5	Ditto	8.82	53.1	16.72	100.3
8	2.0	Ditto	9.18	55.2	16.54	99.2
9	2.25	Somewhat turbid without precipitate.	7.82	47.0	16.00	95.9
10	2.58	Ditto	7.34	44.0	15.00	89.8

Remarks: (1) Filtration of samples numbered more highly than 3 proceeded only very slowly.
(2) Total volume of sample No. 10 was adjusted to 250 *cc* by the addition of 140 *cc* of 5 *M* NaCl. The solubility data involved are of recalculation.
(3) Total N was estimated to be 16.62 ($\pm$0.30) *mg*/100 *cc*, the average value of samples No. 1—No. 9; it was found on sample No. 10 to be 13.34 *mg*/100 *cc*, or 16.68 *mg*/100 *cc* on recalculation.

duplicate) of the filtrates. On the other hand, dispersed nitrogen if infilterable was also estimated, using aliquote portions of the controls, on the supernatant liquids obtained by treating them with a centrifuge. Total nitrogen was estimated on other aliquot portions of the controls kept under agitation.

The results of experiment are described in Table 13 in which the solubilities of eumyosin in buffered NaCl solutions at varying concentrations are given, as estimated after filtration on the one hand and after centrifugation on the other hand. The solubilities are also expressed in the proportions of these estimations in percent of total nitrogen, and illustrated in Figure 7.

Discussions. As it is seen in Table 13 and Figure 7, if we measure the solubility of eumyosin as determined on the centrifuged sols, a remarkable salting-in is concluded; but if it is measured on the filtered sols, another conclusion might be reached that eumyosin is salted-in only to an extent of some 50%. What does account for this discrepancy?

Judging from the extremely slow rate of filtration of the samples as well as from their stability against the centrifugal force, it might be considered that eumyosin particles dispersed in an NaCl solution possess more or less extended fibrous configurations and are more or less strongly hydrated. As a result of strong hydration, they may be sufficiently stable against not too large a centrifugal force. But, owing to their asymmetry in configuration, they may only hardly get through the pores of filter paper, clogging them until a sort of ultrafilter membrane is built up. After such a condition has once been established, only the dispersion medium can pass through the filter so as to concentrate more and more the sol remaining above the filter until the latter sets eventually into a film of jelly as actually observed. This gradual clogging of filter pores may account for the apparent low salting-in of eumyosin as estimated on the filtrates; this suggests, furthermore, that the length of fibrous particles of eumyosin would be of the order of $1 \sim 0.1\ \mu$ which corresponds approximately to the width of filterpores. The insignificant discrepancies in low NaCl concentration range might also support the above reasoning.

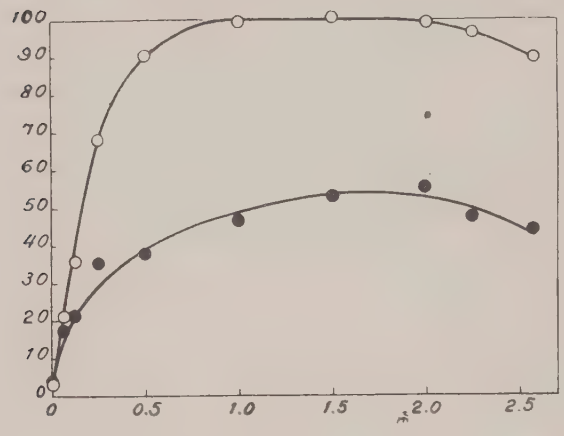


Fig. 7. Solubility of eumyosin in NaCl solutions at pH 7. Abscissa: concentration of NaCl in *mol/liter*. Ordinate: Dissolved N in per cent of total N. Open circles: For centrifuged mother liquors. Solid circles: For filtered mother liquors.

It is detected that the salting-in estimated for the centrifuged samples attains to 100% as soon as NaCl concentration rises to 1 *M*, and is retained to be 100% up to 2 *M* beyond which the salting-out by NaCl takes place more or less explicitly. This suggests that accordingly far much larger figures of solubility would have been obtained than are recorded in the sixth column of Table 13 if samples containing more eumyosin were used in the measurement.

Fresh eumyosin, differing from pseudomyosin, seems to be practically insoluble in water whose pH is controled with a trace of NaOH to be 7.0 (*cf.* Table 10). The minute solubility detected on sample No. 1 might be due to the salting-in action of the phosphate buffer present (0.30 by ionic strength). Eumyosin sols in NaCl solutions appear more or less strongly opalescent and behave as viscous and difficultly-filterable. Those in NaOH solutions are almost limpid with a faint yellowish tinge and can be in most cases rather easily filtered, while those in HCl solutions appear colorless but hazy behaving fairly consistently.

Whereas some quantitative theories have been informed about the solubility of idealized protein molecules (*cf.* CONE and EDSALL, 1943), the origin of salting-in phenomenon remains still somewhat of chemical specificity except that salt-

ing-in bears on the lyotropic series of HOFMEISTER and may be assigned to the formation of amphisalt such as those which PFEIFFER (1923) has pointed out. At any way, in moderately concentrated salt solutions, the hydration of eumyosin is considered to be enhanced so that eumyosin particles go dispersed into the watery mediums; and the abovementioned difference in appearance between several types of sol seems to make some contributions to inferring the feature of eumyosin dispersoids in these sols.

III, 2, 5. Solubility in NaCl Solutions containing HCl or NaOH

Since the salting-in of protein is known to depend on the decrease in its activity coefficient caused by the interaction between the ions of protein and those from the salt, while the ionization of protein, in turn, depends upon the pH of medium, it may be predicted that the features of dissolution of eumyosin in NaCl solutions are altered as the pH's of the solutions are changed. It is known that classical myosin does not dissolve in HCl solutions containing a salt, whereas it dissolves in such in the absence of the latter (BATE-SMITH, 1937; *cf.* also BAILEY, 1944). In other words, the dissolution of myosin in salt solutions is inhibited by the presence of acid. Such inhibition by base may be also probable; but the matter in this connection seems to have been scarcely reported. The action of acid or base involved here cannot be looked upon as salting-out, for a weak acid or basic reaction is sufficiently effective. It cannot also be regarded as an affair of constancy of solubility product, since any acid or base such as that has no common ion to the salt is effective too. It might be considered that acid or base promotes the onesided ionization, *i.e.*, the onesided disionization so as to increase net change, effecting the withdrawal in dipolar moment of the protein; and this weakens the interaction between protein and salt, depressing the salting-in of the protein. The reason why eumyosin remains dissolved in salt solution whose reaction is slightly alkaline seems to be accounted for by the view that the strong dissociation of the ammonium groups on its molecules at such a reaction may certainly keep the molecules virtually in a dipolar state.

Since salting-in as well as *Kamaboko*-forming ability constitute the most distinct characteristic inherent to eumyosin, it may be predicted that the change in the

Fig. 8a—8f. Semiquantitative illustrations of the solubilities of slightly degraded eumyosin in NaCl solutions at various pH's. Abscissa: Concentration of NaCl in *mol/liter*. Ordinate: Dissolved protein in per cent of total protein.

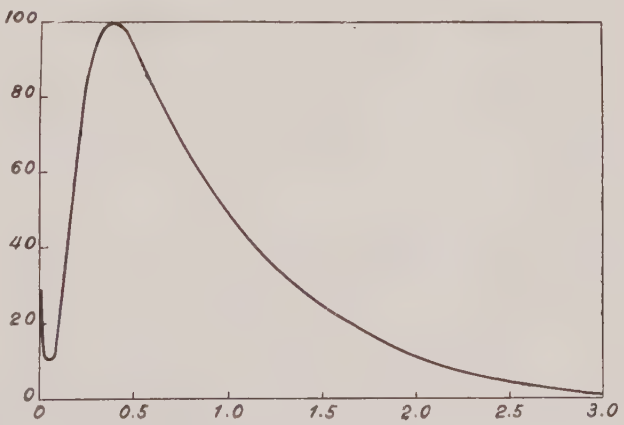


Fig. 8a. at pH 8

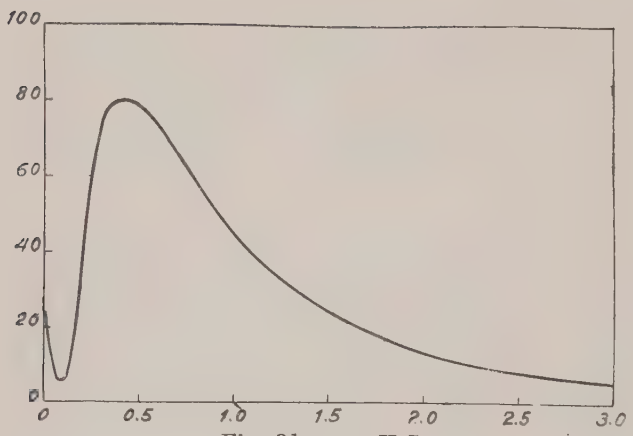


Fig. 8b. at pH 7

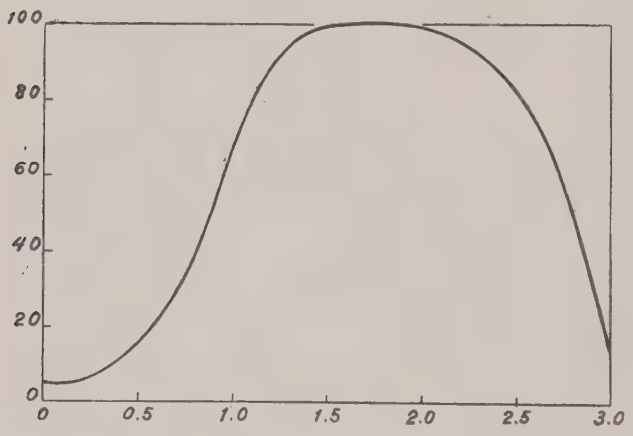


Fig. 8c. at pH 6

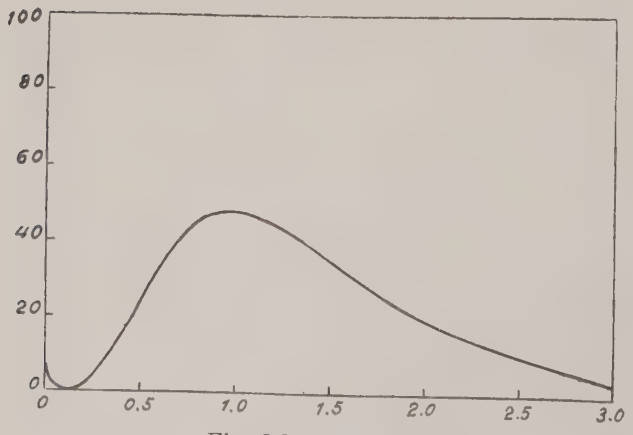


Fig. 8d. at pH 5.5

former property affects directly the latter. Therefore, some substantial data on salting-in of eumyosin in the pH ranges other than neutrality are hoped to be obtained.

There are visualized in Figures 8a–8f the results of semiquantitative investigations on the relative solubilities of eumyosin in several samples which were prepared so as to be constant in protein content and pH but varied in NaCl content. The pH adjustments were done with a dilute solution of HCl or NaOH under controlling the pH-drift towards the isoionic reaction caused by the coexistence of NaCl. In these illustrations, however, it should be noticed that the eumyosin used in these investigations were not free from the effects of aging which had been brought about by preserving the protein at a weakly basic reaction, pH 10.3, for different durations.

It is evident in these figures that the characteristic salting-in of eumyosin, which occurs usually in a moderate concentration range of salt, can be detected only with a few cases where faintly acid reac-

tions of pH from 5.5 to 6.0 are prevailing. The marked salting-in at pH 8.0 and 7.0 is suddenly intercepted due to the defficiency in dipolar moment of eumyosin at these pH's as soon as the salt concentration becomes higher than approximately 0.5*M*; while, at pH 4.5, the high solubility attained due to positive net charge is aboilished even more readily on the similar account when the salt concentration is increased over only 0.1 *M*.

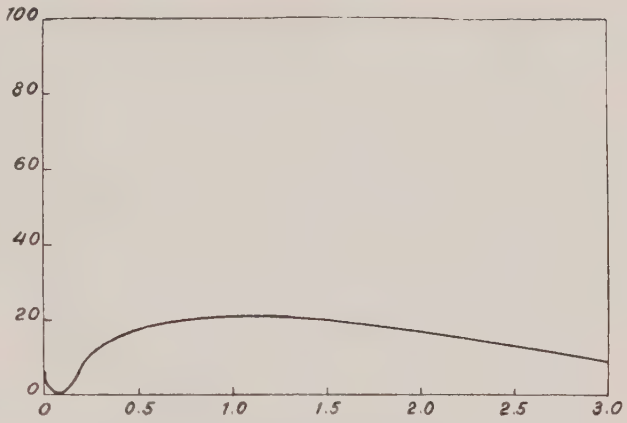


Fig. 8e. at pH 5.0

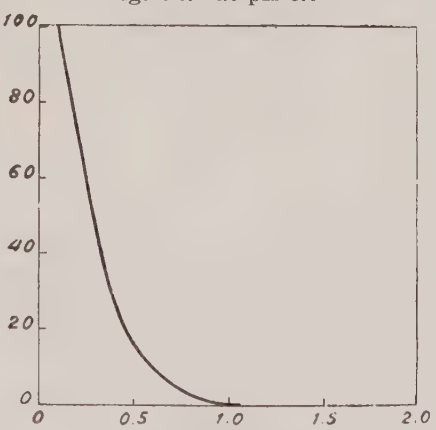


Fig. 8f. at pH 4.5

At very low concentrations of NaCl (lower than 0.1 *M*), a reversion in solubility is detected to occur more or less distinctly forming a minimum presumably in so far as the reaction of the system is alkaline to the isoelectric point (of the somewhat degraded eumyosin in this case). This minimum formation might be ascribed to the contamination by metaeumyosin having been produced during the storage of the stock eumyosin in rather a strong NaOH solution. The fact that the NaCl concentration where the said sort of minimum appears is shifted toward a lower value as the pH is increased from 7 to 8 may be accounted for by that the dipolarity of metaeumyosin is presumably diminished at the same time so as to increase the net charge, thus rendering metaeumyosin more sensible to the effect of coexistent salt.

Speaking generally, the salting-in of eumyosin in NaCl solutions is always suppressed by the addition of HCl or NaOH. So it seems to be probable that *Kamaboko* would not be formed by adding common salt to fish if there is so much acid or alkali in the fish as makes its reaction distinctly acid or basic.

III, 2, 6. Some Additional Observations on Viscosity

Viscosity-concentration relation. According to EDSALL (1930), it seems that ARRHENIUS' empirical formula is applicable to the viscosity of classical myo-

sin dissolved in a salt solution. The same relation has been found to hold for the correlation between the viscosity and the concentration of eumyosin dissolved in a dilute solution of NaOH.[24]

Table 14 contains one of the experimental results obtained with rather a concentrated sol of eumyosin as the starting material, which had been prepared by treating a fresh precipitate of isoionic eumyosin with 0.1 *N* NaOH. Viscosity was measured on the original sol, a sol obtained by diluting the former two times, and such obtained by diluting the same four times. Distilled water was used as the diluent; but pH remained virtually unchanged, *i.e.*, about 9.0 throughout the three samples, suggesting the marked tendency of sodiumeumyosinate to undergo hydrolysis.

Table 14. Viscosity-Concentration Relation of NaOH-Eumyosin Sols. (Temp. 25°C; pH 9.0)

Number of sample	Protein concentration in *gram* per 100 *cc* (C_p)	Relative viscosity (η_R)	$log\ \eta_R$	$\frac{log\ \eta_R}{C_p}$
1 (Original sol)	0.1310	1.977	0.2960	1.13
2	0.0655	1.406	0.1480	1.12
3	0.0328	1.186	0.0740	1.13
			Average	1.13

Temporal mutation of viscosity in NaOH solutions. Viscosity of eumyosin dissolved in an NaOH solution, except in certain special cases, decreases more or less rapidly with the time elapsed; concequently, the more rapidly in the more concentrated NaOH solution and at the higher temperature. Since this viscosity decrease was found to follow the course of a reaction of first order as shown in Table 15, it seems likely that eumyosin particles dispersed in an NaOH solution are changed gradually into simpler units. The nature of the endproduct, however, is supposed to be not very simple. The increase in formol-titrable amino groups was not necessarily positive. The endproduct might possibly be a sort of so-called derived protein such as alkali-protein, proteose, or albumose in a certain case, but it may also possibly be molecular eumyosin resulted from the dissociation of micellar eumyosin in the other case, especially when the descension proceeds at an appropriately low temperature. It remains thus unknown on what factors characteristic to eumyosin is dependent the velocity constant of the viscosity decrease, though the latter seems to be influenced more profoundly by the pH than by the temperature of the sol.

In certain cases, especially when the protein concentration is sufficiently

24) Therefore, as long as ARRHENIUS' equation is applicable, the viscosity of eumyosin may be criticized in the expression $(log_{10}\ \eta_R)/C_p$ which is proportional to the weight intrinsic viscosity (*cf.* KRAEMER (1938)) normally defined as $\lim_{C_p \to 0}\left(\frac{\eta_{sp}}{C_p}\right) = \frac{\log e\ \eta_R}{C_p}$ where C_p is the concentration of anhydrous eumyosin represented in grams per 100 *cc* of solution.

high and the basicity is sufficiently low, it has been experienced that the viscosity of eumyosin sol rises up to an enormous value as shown in Table 16.

Table 15. Temporal Decrease in Viscosity of NaOH-Eumyosin Sols.

Time of measurement	Time elapsed after the first measurement (t) *min*	Time of flow for 10 *cc* (τ) *sec*	$\tau-\tau_\infty$	$log\frac{\tau_0-\tau_\infty}{\tau-\tau_\infty}$	$0.4343t$	Velocity constant $k=\frac{1}{0.4343t}log\frac{\tau_0-\tau_\infty}{\tau-\tau_\infty}$
a: Eumyosin-preparation No. 9; Soluble part at pH 7.5; Temp. of measurement=60°C						
Nov. 30, 15h 57m	0	111.6(=τ_0)	29.9	—	0	—
16h 6m	9	111.0	29.3	0.0088	3.91	0.00225
11	14	110.6	28.9	0.0148	6.08	0.00234
16	19	110.4	28.7	0.0178	8.25	0.00216
21	24	110.1	28.4	0.0224	10.43	0.00214
40	43	108.7	27.0	0.0443	18.68	0.00237
17h 5m	68	107.7	26.0	0.0519	29.54	0.00176
33	96	106.3	24.6	0.0848	41.40	0.00205
18h 10m	133	103.6	21.9	0.1353	57.78	0.00234
36m	159	102.2	20.5	0.1639	69.1	0.00234
19h 1m	184	101.6	19.9	0.1768	79.9	0.00222
20h 16m	269	97.5	15.8	0.2270	116.9	0.00237
Dec. 2, 9h 23m	2496	81.8	0.1	—	—	Average
10h 3m	2536	81.7(=τ_∞)	0.0	—	—	=0.0022
b: Eumyosin-preparation No. 11: pH of the Sol=10.8; Temp of measurement=60°C						
13h 27m	0	238.6(=τ_0)	41.6	—	0	—
32	5	233.1	36.1	0.0616	2.17	0.0284
38	11	229.5	32.5	0.1072	4.77	0.0235
43	16	225.4	28.4	0.1658	6.95	0.0239
47	20	220.3	23.3	0.2517	8.69	0.0290
51	24	219.7	22.7	0.2631	10.43	0.0252
55	28	217.8	20.8	0.3010	12.16	0.0248
14h 9m	48	210.4	13.4	0.4920	18.2	0.0270
18	51	210.0	13.0	0.5025	22.1	0.0229
27	60	207.4	10.4	0.6021	26.0	0.0230
37	70	206.3	9.3	0.6506	30.4	0.0214
41	74	205.3	8.3	0.7000	32.1	0.0218
45	78	203.5	6.5	0.8062	33.9	0.0238
49	82	201.5	4.5	0.9659	35.6	0.0268
58	91	200.8	3.8	1.0393	39.5	0.0263
15h 7m	100	199.1	2.1	1.2969	43.3	0.0300
16	109	198.8	1.8	1.3638	47.3	0.0288
25	118	198.6	1.6	1.4050	51.2	0.0275
34	127	198.1	1.1	1.5777	55.1	0.0287
47	140	198.0	1.0	1.6191	60.8	0.0267
16h 2m	155	197.8	0.8	1.7160	67.3	0.0255
18h 30m	303	197.1	0.1	2.6191	131.6	0.0199
20h 0m	393	197.0(=τ_∞)	—	—	—	Average
						=0.026

But in such cases too, the increase in viscosity is always preceded by an incipient decrease in viscosity. Even when the basicity of sol is low, such a conspicuous rise in viscosity never takes place if its eumyosin concentration is low; and even when the protein concentration is high, the peculiar rise in viscosity never occurs if the basicity is high. So it might be inferred that, while there prevails in the early stage quite the same motive causing the disintegration of

eumyosin particles as in other general cases, the motive causing later the striking rise in viscosity should be quite different in nature from the disintegration motive. This means that, in other words, at least *two kinds of force* may be conceivable, which can raise the viscosity of eumyosin, presumably by causing it to form larger particles. The phenomenon of peculiar increase in viscosity seems to be related intimately to the phenomenon that the characteristic clotting of eumyosin takes place at pH 8.5, which has been put to use by the author in his procedure for *Kamaboko*-formable eumyosin preparation and will be treated later on more in detail (*cf.* IV, 3).

Table 16. An Example of Temporal Increase in Viscosity of NaOH-Eumyosin Sol.
Protein concentration = 0.679 $g/100\,cc = C_p$
pH = 9.85; Temperature of measurement = 25°C.

Time of measurement		Relative viscosity η_R	Intrinsic viscosity $log\,\eta_R/C_p$
Apr. 10,	$17^h\ 50^m$	3.080	0.720
	55	2.920	0.686
	$18^h\ 0^m$	2.869	0.674
	6	2.850	0.667
	12	2.832	0.666
	56	2.817	0.663
	$19^h\ 9^m$	2.782	0.655
	15	2.920	0.686
	20	2.988	0.701
	26	3.110	0.726
	33	3.400	0.783
	40	3.868	0.866
	48	3.982	0.885
	56	4.252	0.926
	$20^h\ 4^m$	4.394	0.947
Apr. 11,	$8^h\ 55^m$	6.958	1.242
	$9^h\ 13^m$	19.70	1.907
Apr. 12,	$9^h\ 17^m$	1.273	0.155

The remarkable decrease in viscosity which was observed at the end of the measurements, when the sample was found to be putrid, may be accepted to be due to the putrifactive disintegration of eumyosin particles.

III, 2, 7. Sedimentation at Various pH's

As often mentioned, eumyosin dissolved in a highly diluted solution of NaOH forms at a pH of about 8.5 characteristic colonies of flocculum which are so large in size that they settle rapidly even while the mother liquor is still swirling. This characteristic colony formation at pH 8.5 seems to be very analogous to the formation of raw *Kamaboko*-jelly in the respect that both these phenomena take place under such a condition as that where just a partial dissolution of eumyosin occurs, yielding highly viscous sols. It seems, therefore, to be of major importance for the interpretation of *Kamakoko*-formation phenomenon to investigate more fully the nature of this colony formation.

Experimental. An eumyosin sol at a pH of about 10, which had been obtained by dissolving a fresh preparation of eumyosin in an NaOH solution, was used as the stock solution in this experiment. Every 500 *cc* of the stock solution were added with varying amounts of 0.1 *N* HCl and of distilled water

so that a series of samples having been different in pH but equal in total volume (550 *cc*) were provided as shown in Table 17 a.

Table 17 a. Appearance of Eumyosin Flocculum prepared at Various pH's. (Samples were prepared in conical beakers; *cf.* also Table 17 b.)

Number of sample	0.1 *N* HCl added (*cc*)	Distilled water added (*cc*)	pH	Appearance	
				Deposit of flocculum	Mother liquor
1	2.0	48.0	9.4	Greenish, bluish white.	Bluish, semitransparent; looked like wet frosted glass.
2	2.5	47.5	9.2	Greenish, bluish moderately fine colonies.	Perfectly clear.
3	3.0	47.0	8.9	Greenish blue.	Faintly turbid with bluish tinge.
4	3.8	46.2	8.4	Tinged with greenish blue.	Almost perfectly clear.
5	4.6	45.4	8.0	Bluish white, semitransparent.	Water-clear.
6	5.4	44.6	7.6	Ditto	Perfectly clear.
7	6.8	43.2	7.2	Greyish white, semitransparent.	Looked like wet flosted glass.
8	8.5	41.5	6.8	White, tinged pink.	Perfectly clear.
9	9.5	40.5	5.9	White, semitranparent.	Ditto
10	10.0	40.0	5.4	White, tinged pink; cirrus-like.	Ditto
11	10.2	39.8	5.3	Opaquely white.	Water-clear.*
12	11.5	38.5	4.8	Bluish white, semitransparent; fine colonies.	Slightly turbid.
13	12.0	38.0	4.5	Silvery grey, semitransparent.**	No trace of supernatant liquor became separated.

* The appearance as well as the pH of this mother liquor remained unchanged over several days. On the addition of H_2SO_4, the liquor became turbid. The turbidity did not form flocculums. It vanished on heating the system but reappeared on cooling. It disappeared when stirred, and reappeared when allowed to stand. The nature of this turbidity remains still unknown. Such turbidity did not occur if sulfosalicylic acid was used instead of H_2SO_4.

** Though the flocculums did not agglomerate for a long duration, they did not get through filter paper on filtration, separating a clear leach.

Every 500 *cc* of these somples having been homogenized by being stirred up were put into every one of a series of glass-stoppered measuring cylinders. The cylinders were then stoppered, turned gently several times so that their contents might be homogenized, and allowed to stand quietly. The plot of the head of sediment layer against the time elapsed assumed an inverted italic *S*-like shape whose inclination was varied among different samples. The maximum descension rate attained at the inflexion point on the plot for each sample is listed as its sedimentation velocity in Table 17 b. As sediment volumes, those values are listed, which were arrived at when the heads apparently ceased to sink further. Although it was experienced that the sediment volumes thus defined shrank afterwards more or less appreciably as a result of syneresis, such apparent final values of sediment volume were adopted, for certain other obstacles, *e.g.*, the floating up of deposit, *etc.*, took place afterwards in several samples. The

Table 17 b. Sedimentation Velocities, Sediment Volumes, and Solubilities of Eumyosin at Various pH Values. (12-13°C)

Number of sample	pH	Maximal sedimentation velocity (*mm/min*)	Sediment volume (*cc*)	N dissolved in mother liquor (*mg*/100 *cc*)	Dissolved N in *per cent* of total N	Remarks of the behavior of deposit layer
1	9.4	?*	50-495	1.970	11.90	Weakly jelly-forming; but a part of the deposit was readily swirled up like dust.
2	9.2	5.8	57	1.676	10.03	A mass of jelly which behaved very flexibly.
3	8.9	10.0	51	1.080	6.46	A thixotropic mass of jelly which was so plastic as to be wound up into a ropy shape on gentle swirling.
4	8.4	10.5	56	0.638	3.82	A tough mass of jelly which was not easily poured out.
5	8.0	5.6	120	0.293	1.75	Somewhat flocculant; the head of sediment layer was decided only with difficulty.
6	7.6	0.52	160	0.241	1.44	The head was readily decidable.
7	7.2	1.24	182	0.215	1.29	
8	6.8	3.88	167	0.084	0.53	
9	5.9	12.7	106	0.077	0.46	
10	5.4	19.1	97	0.028	0.17	
11	5.3	17.9	100	0.051	0.31	
12	4.8	0.93	150	0.045	0.27	Somewhat flocculant; the head was decidable only with difficulty.
13	4.5	0	500	?	?	No separation of mother liquor.

* In sample No. 1, the flocculation of emyosin proceeded so hesitatingly that the flocculums deposited only little by little to the bottom. There were formed finally three layers of deposit in the cylinder, of which the top layer was water-clear, the middle was hazy and somewhat diffused at the top, and the bottom layer was dense deposit tinged bluish and had an exactly decidable head.

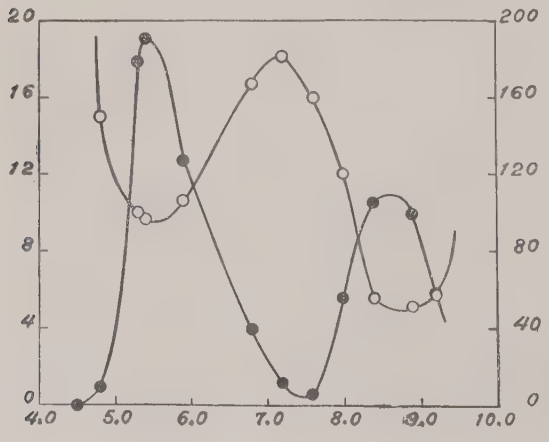


Fig. 9. Sedimentation velocity and sediment-volume of eumyosin flocculum at various pH's. Abscissa: pH of mother liquor. Ordinate: Sedimentation velocity in *mm/min* (left); sediment volume in *cc* (right). Open circles: Sediment volume. Solid circles: Sedimentation velocity.

sedimentation velocity and the sediment volume defined above are shown in relation to the pH of system also graphically in Figure 9.

From Figure 9 in which the curve for sediment volume runs almost antipodally to that for sedimentation velocity, it could be suspected that the virtual volume of the flocculum itself would become minimal not only at pH 5.5 but also at 8.5. However, if eumyosin is precipitated under a strong centrifugal force as will be described in III, 2, 8, the minium in sediment volume detected at pH 8.5 becomes far less conspicuous or even vanishes (*cf.* Figure 10). This transformation in sediment volume-pH curve caused by centrifugation will be discussed in the following section, III, 2, 8.

At any way, it is suggested again the *duality* in the force responsible for the colony-formation of flocculums.

III, 2, 8. Influence of pH on the Sediment Volume under a Strong Centrifugal Force

As described in the preceding section, the sediment volumes at an apparent equilibrium given in Table 17 b were found to become subsequently more or less contracted during a long period of time.

Table 18. Sediment Volumes of Eumyosin centrifuged at Various pH's.

Number of sample	pH	Water content of the deposit in weight per cent	Relative sediment volume. (Volume of water per unit weight of anhydrous eumyosin)*	Remarks on the behavior of the centrifuged deposit
		%	*cc*	
1	9.4	99.60	249	Very voluminous; three layers were formed; syneresis occurred.
2	9.2	99.06	105	A voluminous mass of jelly; syneresis occurred.
3	8.9	98.71	76.5	A voluminous, bluish, semitransparent mass of jelly; deformed on declining the cup, but held remaining in the turned cup.
4	8.4	98.58	69.5	
5	8.0	98.62	71.5	
6	7.6	98.75	79.0	Greenish blue, semitransparent; voluminous; paste-like without spinnability.
7	7.2	98.68	74.1	Moderate in bulkiness; white but semitransparent; flowed on declining the cup.
8	6.8	96.19	25.2	Compact; milky white; did not flow on declining the cup.
9	5.9	95.65	22.0	Compact; milky-glass-like; did not flow on declining the cup.
10	5.4	95.56	21.5	Very compact; opaquely white; was not deformed on declining the cup.
11	5.3	95.70	22.2	Ditto
12	4.8	98.98	97.0	Only a small amount; milky white; paste-like slack jelly.
13	4.5	—	—	Sol state.

* No correction has been made against the effects of electrostriction, *etc.*

Hereupon, in order to find sediment volumes such as would be far closer to those in true equilibrium, the eumyosin deposits were centrifuged, and their

water-contents were measured as stated below. In such a water-rich system as eumyosin deposit, it may well be assumed that the figure of the water-content per unit weight of anhydrous matter expressed in *gram* represents approximately the sediment volume in *cc* per unit weight of anhydrous matter. The remainders of the samples on which all the treatments necessary to provide the data mentioned in III, 2, 7 had been completed were homogenized by stirring, and a portion of each one of the homogenized suspensions was treated by an ordinary centrifuge driven by a motor; and the water-content of each centrifuged deposit was determined in a usual manner by drying it up at about 100°C. The figures thus obtained are expected to be nearly parallel to the sediment volumes in true equilibrium. They are listed in relation to the pH of mother liquor in Table 18 and also shown graphically in Figure 10.

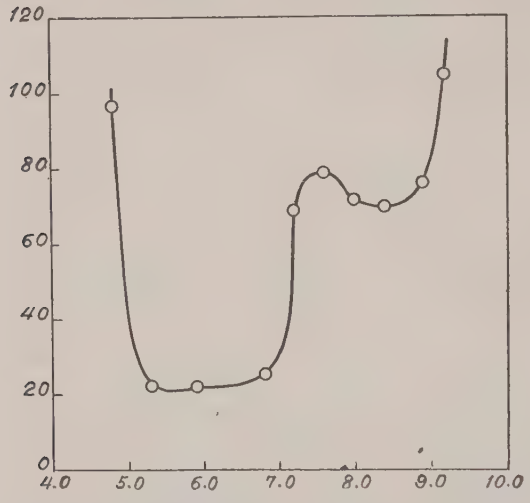


Fig. 10. Sediment-volumes of eumyosin under a strong centrifugal force in relation to pH. Abscissa: pH of mother liquor. Ordinate: Sediment volume represented in *cc* of water per unit gram of anhydrous protein contained in the deposit.

Comparing Figure 10 with the sediment volume-pH curve in Figure 9, it is seen that the centrifugation has brought about some remarkable changes in sediment volum: (1) The valley in sediment volume, previously situated in a relatively narrow zone of pH around the isoionic point (pH 5.5), has become broadered, extending itself up to pH 7. (2) The second minimum at pH 8.5 has almost vanished, while only a trace of it still remains at the foot of the salient portion of the curve, which has been retained in the vicinity of pH 7.5.

Discussion. Now that the curve shown in Figure 10 may well be regarded approximately as the equilibrium sediment volumes in the pH range extending from 4.5 to 9.5, the state of accumulation of eumyosin particles within the centrifuged deposit should be contemplated standing on the basis of the feature of this curve and also referring to other data available:

As the sediment volume of a colloid varies in most cases in parallel to its hydrophilicity, it will be fruitful to compare the curve in Figure 10 which is related to the saturating body with that in Figure 11 (or Figure 4) which represents the solubility of eumyosin, *i.e.*, the extent of hydrophilicity in liquid phase. If we imagine either one of Figures 10 and 11 lapped over the other and scrutinize the distinction between these two curves, we may arrive at the conception that the protuberance found at the pH around 7.5 on the former should represent quite a peculiar property belonging only to the deposit phase, *i.e.*, a sort of *concentrated system.* This means, in other words, that there should be

an important factor available for the formation of the characteristic gelatinous deposit of eumyosin at pH 8.5, which would be quite different from simple interionic COULOMB forces, whereas the latter may affect according to MEYER (1929) the configuration of individual molecules themselves and also may contribute to the electrokinetic potential of eumyosin particles.

The discussion on the nature of this jelly-forming force will be reserved here. It should be noticed, however, that the peculiar salience extending from pH 7 to 9 on the sediment volume curve in Figure 10 suggests that the eumyosin particles precipitated in this critical zone of pH, in whatever degree of intermolecular association they may have been constructed, assume remarkably elongated configurations so that far greater bulkiness may be attained when they accumulate with random orientation, in comparison with those in the isoionic zone.

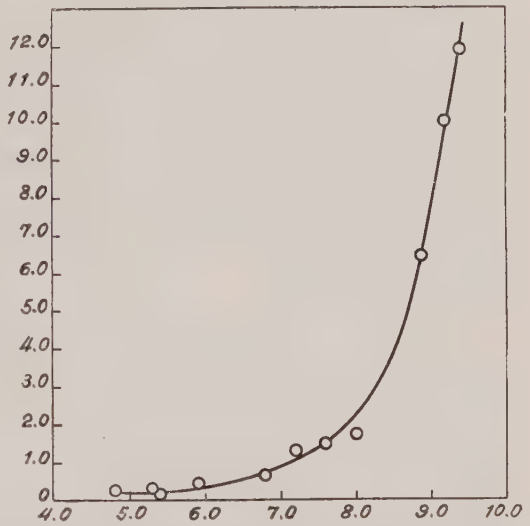


Fig. 11. Solubility of eumyosin in HCl or NaOH solutions at various pH's. Abscissa: pH of mother liquor. Ordinate: Dissolved N in per cent of total N.

Referring to the amino acid composition of classical myosin (*cf.* BAILEY, 1944), the electrolytic dissociation of eumyosin as a cation may be analogized from the dissociation constants of the constituent dicarboxylic amino acid so as to be completed in a narrow pH range a little acid to the isoionic point. In this pH range, eumyosin carries no longer negative charges available to cancelling the ionization of cationic groups, which thus would have already been completed. Keeping this in mind, if we remember the fact that the characteristic phenomenon of jelly-formation of eumyosin can be detected only in a basic pH range adjacent to the isoionic zone, we come to a provisional conclusion that: At least one of the necessary conditions for the characteristic jelly-formation, and hence also for the *Kamaboko*-jelly-formation, which is presumed to be brought about through a very analogous mechanism, seems to be likely that eumyosin exists in such a special condition as that where its molecules are furnished with plenty of total charge as well as of net charge at the same time.

Remarks 2. Though the chemistry of muscular contraction has recently made jumping advances since the discovery of the extremely important function of adenosinetriphosphate (*cf.* ENGELHARDT, 1949; SZENT GYÖRGYI, 1947), it may still be significant the result obtained in III, 2, 8, which implies that eumyosin particles can be contracted by a minute depression, *i.e.*, without any appreciable change in pH, in the narrow critical zone of pH 7.0–7.4 which agrees with the physiological reaction of muscular system *in vivo*.

III, 3. Spontaneous Irreversible Changes

It has often been reported that classical myosin is readily disintegrated when treated with a solution of strong base, whereas the present author has proposed as a procedure to prepare eumyosin the application of an NaOH solution at ice-cold or so. Even in the absence of such a drastic agent, classical myosin has been said to lose the original dispersibility in neutral salt solutions if it is kept flocculated for a long period of time. Although these changes in the properties of myosin, of course, may certainly depend upon the temperature as well as the duration of its storage, they seems to be profoundly affected by the pH of environment, which should be also a momentous determinant for the state of ionization and hence of hydration of myosin.

In this section, it will be described several results of experiments with eumyosin, the earlier members of which are related chiefly to the effects of promoted hydration, while the later members concern primarily the influences of depressed hydration.

III, 3, 1. Effects of Heat on Solubility

It is not always valid to predict that there will take place the flocculation of protein whenever an eumyosin sol is heated. Some sorts of derived protein, soluble in water, such as assigned to metaprotein or albumose, *etc.*, might be produced under certain circumstances. Similarly, even with the case of eumyosin existing in the state of deposit, it has often been experienced that a part of the deposit is made so as to become dissolved in the mother liquor. In order to apprehend the general feature of heating-effects, if such prevails throughout the extensive thermal changes of eumyosin, some details of experiments will be given in the following.

III, 3, 1, 1. Thermal-effect on the solubility of eumyosin contained in neutral salt solution

When eumyosin is heated in a neutral salt solution, its solubility is changed irreversibly to a larger or smaller extent depending on the concentration of the salt.

For example, while a set of subdivisions of an intact preparation of eumyosin were salted-in respectively in variously-concentrated solutions of potassium buffer at pH 7.0 (COHN, 1927)[25] as are shown in the fourth line in Table 19 (samples of series 1) as well as by curve 1 in Figure 12, another set of duplicate subdivisions of the same preparation behaved quite differently in solubility after they were heated at 50°C for an hour in the corresponding buffer solutions, as shown in the fifth line in the same table (samples belonging to series 2) as well as by curve 2 in the said figure. Comparing curve 2 with curve 1, it is seen that, when the salt coexists with eumyosin at concentrations higher than 0.1 M (0.6_4^1 by ionic strength), the protein is modified by heat so as to become

25) With aqueous solutions of this salt mixture, the figures representing the concentration by ionic strength are always 6 times as large as those represented by molal concentration in independence on the pH of the solutions.

more or less appreciably insoluble, while it becomes soluble when heated under poor coexistence of the salt as low as 0.05 *M* (0.3 by ionic strength).

Table 19. Heat-effect on the Solubilities of Eumyosin contained Variously-Concentrated Potassiumphosphate Solutions.

Number of sample		1	2	3	4	5	6	7	8
Concentration of the phosphate	*mol*	0.05	0.10	0.143	0.30	0.50	0.75	1.00	1.50
	ionic strength	0.30	0.60	0.86	1.8	3.0	4.5	6.0	9.0
Filterable N in per cent of total N	Series 1	12.1	94.4	96.5	95.5	95.7	94.8	93.0	17.2
	Series 2	39.2	87.6	84.0	72.6	59.5	34.5	11.2	6.5
	Series 1′	90.6	93.8	96.3	85.2	80.7	79.0	78.2	9.7
	Series 2′	93.1	91.3	90.5	88.3	82.5	68.0	16.4	9.9

On the other hand, if eumyosin is heated beforehand in a salt solution in which the protein has been substantially salted-in, it recedes in heat-coagulability over a broad concentration range of salt coexistent with it when it is heated again. Such situation is illustrated in the sixth and seventh lines in Table 19 and also by curves 1′ and 2′ in Figure 12. In the experiment, an eumyosin sol having been prepared in 0.5*M* potassium phosphate buffer was heated previously at 100° C for an hour and subdivided afterwards into two sets of samples which were adjusted to be identical in protein-concentration but variant in salt-concentration. The solubilities of the heat-attacked eumyosin were estimated directly with one of these sets of samples (series 1′), while its heat-coagulabilities were determined with the other set which were heated additionally at 50°C for an hour (samples of series 2′).

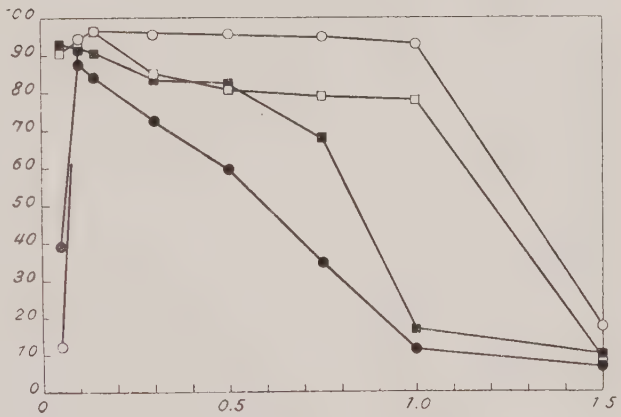


Fig. 12. Thermal-effect on the solubility of eumyosin contained in neutral salt solution. (*cf.* the text.) Abscissa: Concentration of potassium phosphate in *mol/liter*. Ordinate: Dissolved N in per cent of total N. Open circles: Curve 1. (Before heating.) Solid circles: Curve 1′. (After heating at 50°C for an hour.) Open squares: Curve 2. (Solubilities of eumyosin previously heated at 100°C for an hour *i. e.*, heat-affected eumyosin.) Solid squares: Curve 2′. (Solubilities of the heat-affected eumyosin additionary heated at 50°C for an hour.)

Comparing curve 1′ and 2′ with each other, it is seen that eumyosin heated previouly in 0.5 *M* phosphate buffer at 100°C for an hour can still undergo additional coagulation even at 50°C, far lower a temperature, if the additional heating is carried out in a buffer solution more concentrated than that in which the protein has been heated previously, suggesting that the ionic strength of the

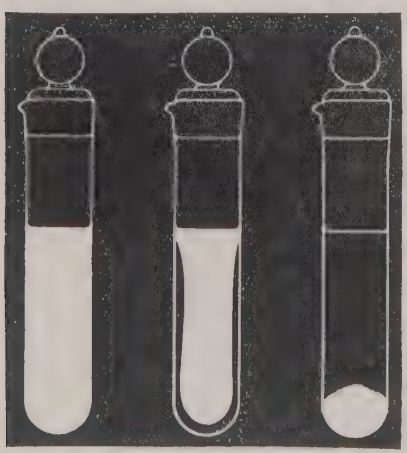
medium and hence the state of ionization of eumyosin itself may certainly be the primary determinant for its heat-coagulability compared with the temperature.

Next, comparing curve 2′ with curve 2, it is understood that the heat-coagulability of eumyosin at 50°C is reduced more or less appreciably if the protein is previously heated in 0.5 *M* buffer at 100°C.

Finally, if curves 1, 2 and 1′ are compared with one another, it is concluded that, when eumyosin contained in 0.5 *M* phosphate buffer at pH 7.0 is heated, despite that a considerable portion of the protein is coagulated at 50°C, only a far smaller portion is coagulated at 100°C. This points to that a larger or smaller portion of heat-coagulum of eumyosin once formed at a relatively low temperature is changed into a soluble matter consisting presumably of far smaller units on being heated at higher temperatures exceeding a certain critical value just like the wellknown phenomenon that insoluble collagen is hydrolysed by hot water into soluble gelatin.

Eumyosin sols having contained the phosphate only at concentrations lower than 0.5 *M* did not give any coagulum large enough to be detected by naked eye, even though some of them became strongly opalescent on being heated. Even in those having contained the salt at concentrations higher than 0.5 *M*, the formation of heat-coagulum took place rather long after they had reached the specified temperature. For instance, sample No. 6 in series 2′ began to give flocculums first after 20 minutes since it had attained to 50°C, having separated a faintly turbid mother liquor, while sample No. 7 of the same series gave coagulum early in 3 minutes. On the other hand, sample No. 6 in series 2, which was identical in salt content to sample No. 6 in series 2′, contained no flocculum and behaved as a weak thixotropic mass of jelly for a pretty long while after the heat treatment had been completed; but the rigidity of the jelly was so implicit that it was detected only with dfficulty on declining the container with great cautiousness. This thixotropic jelly, however, after a fairly long period of hours, began rather suddenly to shrink, separating an almost water-clear liquid over the inner wall of the container. After the shrinkage had once started, the jelly was readily crumbled into flocculums on gently stirring the system, as sketched in Figure 13.

Fig. 13. Thermal coagulation of eumyosin in a salt solution.



a b c

a: Thixotropic jelly.
b: Syneresis began.
c: Crumbling-down into flocculum.

As will be discussed later (*cf.* IV, 3), emyosin micelles are most favorably fixed to form skeletal frameworks constituting a mass of jelly when their constituent molecules are considerably if restrictedly stabilized by net charge or with the envelopes of hydration water. Therefore, if an eumyosin sol is heated under such conditions, its constituent micelles will be promoted on the one hand to agglomerate

into a mass of jelly,[26] and on the other hand to be dissociated into singular molecules. Furthermore, if the heating is prolonged, especially at high temperatures, the jelly will undergo syneresis followed by flocculation, while the singular molecules will be modified into an incoagulable matter, metaeumyosin. Moreover, as long as the jelly behaves as a thixotropic gel within which the constituent molecules are to be bound together only loosely to form the skeletal micelles, the jelly itself will be forced by thermal agitation to set free some singular molecules from it, which will again be denatured into metaeumyosin. It is likely that whether a major portion of total eumyosin are flocculated or metaproteinized depends not only upon the ionic condition of medium and the temperature of heating but also presumably upon the protein-concentration of the sol being heated. At any rate, the unexpected results observed in the present experiment that the eumyosin sol gave less heat-coagulum at a higher temperature, 100°C, than at 50°C may well be interpreted from the idea just mentioned, *i. e.*, the dual tendency of eumyosin micelles against thermal agitation.

About the origin of this dual tendency of eumyosin micelles and that of the thermal denaturation of its singular molecules, the discussions in IV, 4 should be referred to.

III, 3, 1, 2. Solubility of heat-affected eumyosin in salt solutions

In this experiment, an eumyosin suspension at pH 7, which had been prepared by repeatedly washing the isoelectric precipitate with tap water, was employed as the starting material. In order to provide several groups of suspensions which had previously been affected by heat to various extents, every 20 *cc* of the starting suspension were put into every one of glass-stoppered tubes, added with a few drops of toluene, and kept in an incubator at 40°C, but leaving out a single group as the control. Every several members of these tubes were taken out from the incubator after different periods of storage specified for each group of the suspensions; and the samples for solubility measurement were prepared with these members as follows:

The contents of the tubes were added first with calculated amounts of 5 *M* NaCl; then, their pH's were adjusted to 7.5 with 0.1 *N* NaOH; and finally, they were diluted up to a definite volume (50 *cc*) by the addition of distilled water so that such a series of samples were obtained as were constant in the total concentration of protein and in pH but variant in NaCl concentration. On each one of these samples, after its appearance had been scrutinized, the bulk of precipitate as well as the protein concentration in mother liquor were esti-

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26) SUYAMA (1950-51) observed that the viscosity of a purified myosin sol in neutral salt solution, despite of its regular dependence upon temperature below some 45°C, did not give any reproducible values at higher temperatures above the said. His result seems to imply that the sol is transformed into a thixotropic system above some 45°C, the consistency of which may possibly depend on the degree of breaking-down its spatial structure.

mated in comparison with those of other samples ;[27] and thus the effects of heat on eumyosin were investigated semiquantitatively.

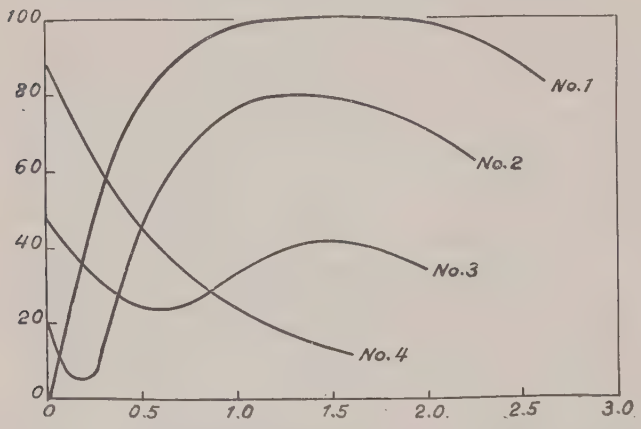

Fig. 14. Solubility of eumyosin in NaCl solutions, which had been attacked by heat to various extents. Abscissa: Concentration of NaCl in *mol/liter*. Ordinate: Relative amount of dissolved protein in semiquantitative scale. The order of the extent to which the protein suffered heat-attack: curve No. 1<curve No. 2<curve No. 3<curve No. 4.

The results are shown schematically in Figure 14. In this figure, curve No. 1 represents the solubility of the control. Curves No. 2 and No. 3 indicate respectively the solubilities of the suspensions which were previously kept at 40°C for 10 days and 30 days; and curve No. 4 shows the solubility of eumyosin which was heated additionally in a boiling water bath for 30 minutes after it had been kept at 40°C for 32 days.

It is seen from Figure 14 that: The solubility of fresh eumyosin is considerable over a wide range of NaCl concentration, attaining to 100% to the approximation between 1 *M* and 2 *M*, while it behaves as insoluble in an extremely dilute solution of the salt (*cf.* curve No. 1, or Figure 7).

When eumyosin has been attacked a little by heat, there appears a minimum solubility at a very low concentration of NaCl, while it behaves as somewhat soluble in plain water; at the same time, the solubility hill in the range of moderate concentrations of NaCl becomes somewhat lowered and narrowed (*cf.* curve No. 2).

With the further advance of heat-effect, the solubility valley is shifted towards the higher concentration of NaCl and also widened and shallowed, while the solubility in plan water becomes more pronounced on the one hand, and that in moderately concentrated NaCl solutions becomes more reduced on the other hand (*cf.* curve No. 3). And thus, when eumyosin is attacked by heat extremely, it becomes completely soluble in plain water and difficultly soluble in NaCl solutions of moderate concentration. Such monotonous changes in solubility as here observed, *i.e.*, the steady increase in plain water and the steady decrease in moderately (or highly) concentrated NaCl solutions, might probably be able to be utilized to estimate quantitatively the extent of heat damage which a sample of eumyosin has suffered.

Then, what a sort of change in the molecular structure of eumyosin

27) The protein concentration in mother liquor was estimated on the strength of flocculation which took place when 10 *cc* of the filtrate of mother liquor were added with 5 *cc* of 10% trichloracetic acid.

caused by heat does give rise to such a change in solubility as mentioned above? Though the molecular structure of myosin has recently become extensively manifested (*cf.* Schmitt, 1944; Bailey, 1944; Astbury, 1944; Tristram, 1944; Pauling and Corey, 1951), there still remain a number of indistinct points in the subject, for instance, the phenomenon of denaturation with which the abovementioned problem may probably be connected. Any conclusive answer, therefore, cannot be given for the present; but some discussions on the problem will tentatively be made in the following.

Discussion. If we compare curve No. 4 in Figure 14 with the curve in Figure 8 f which represents the solubility of eumyosin in acid solutions of NaCl at pH 4.5 where eumyosin exists presumably as a nearly pure cation, there will be found a striking analogy between them. This suggests that the heat applied to eumyosin affects the latter so as to behave in neutral NaCl solutions as if it were a non-dipolar anion. The fact that the solubility hill in NaCl solutions of moderate concentration, *i.e.*, the characteristic salting-in of fresh eumyosin, retires gradually with the advance of heat-effect seems also to support such a suggestion, for as mentioned above (*cf.* III, 2, 5), the characteristic salting-in of eumyosin should be able to occur only when eumyosin possesses plenty of *celibate* positive charges and *celibate* negative charges simultaneously, in other wards, when the protein is strongly ionized into a state of dipolar nature.

Judging from the irreversible growth of the solubility in plain water, it is conceivable that heat causes large micellar particles of intact eumyosin to disintegrate into small particles which are not only sufficiently fine to be dispersed stably against the gravitational force but also deprived of cohering ability into large particles on cooling. The recession in salting-in-ability as well as the liability to salting-out, both of which are effected also irreversibly by heat, suggest that the disintegration product may presumably be configured so as to behave as particles displaying rather a small dipolar moment. The feature of the thermal change of eumyosin micelles and the irreversible change in the properties of their dissociation product will be discussed in detail in the following chapter, IV.

It was experienced, on the other hand, that the behavior of eumyosin having been attacked partially by heat was not entirely the same as that of a mixture of fresh eumyosin and completely damaged eumyosin, whereas a larger or smaller fraction of such partially modified eumyosin, which had been isolated with ease, was ascertained to possess a marked *Kamaboko*-forming ability. This fraction, however, despite of its prominent *Kamaboko*-forming ability, behaved somewhat differently to fresh eumyosin in the respect that it was completely insoluble in dilute solutions of NaCl, *e.g.*, below 0.1–0.2 *M*, and, in addition, it was more readily salted-out than fresh eumyosin by concentrated solutions of the same salt, suggesting the relative largeness in particle size of this fraction. Such results of examination as these point to a fact of major importance that "freshness" is by no means a necessary factor for the *Kamaboko*-forming ability of eumyosin; and they suggest further that the phenomenon of *Kamaboko*-formation might certainly be realized among rather large particles of eumyosin. As it was confirmed further, on the other hand, that eumyosin having

been damaged extremely by heat did not possess even a trace of *Kamaboko*-forming ability, it may well be inferred that eumyosin partially damaged by heat corresponds with a mixture of a *Kamaboko*-forming fraction and a non-*Kamaboko*-forming fraction.[28] The orderly displacement detectable among the curves in Figure 14 from No. 1 towards No. 4 seems to be explained satisfactorily from the point of view mentioned just above.

The reason why some fractional members of eumyosin particles, contrary to the other members, become rather compact on heating as implied by the abovementioned withdrawal in the readiness of salting-in will be discussed later (IV, 4, 2).

III, 3, 1, 3. The solubility-changes of eumyosin contained in acid or base solutions

According to WEBER's (1925 b) study on classical myosin, the protein in a dilute buffer solution is not coagulated by heat on the outsides of a pH range from about 4.5 to about 7. As he states that whole the protein is coagulated so far as any amount coagulates, it might be inferred that, while the protein is completely coagulated at a pH situated only slightly nearer to the isoelectric point than pH 4.5 or pH 7, it is completely not-coagulable at a pH slightly more acid or basic to pH 4.5 or pH 7, respectively. However, this is not the case with eumyosin at least as seen from the behavior of the sample No. 1 shown in Table 19: When an aqueous system containing eumyosin at pH 7 was heated for an hour, various portions of the protein were coagulated according to the temperatures of heating.

This result of experiment suggests that, if eumyosin is heated at different pH's or in differently strong solutions of an acid or a base, where the various portions of the protein are kept precipitated respectively, the portions coagulated by heat are also variant. If this suggestion hits the truth, it may lead to the further conception that the coagulation of eumyosin is directly dependent upon its ionic feature. The experiments carried out in order to ascertain the validity of the suggestion will be described in the following.

Solubility change in weakly acid or basic solutions. A stock sol of eumyosin in a dilute solution of NaOH at pH 10.5, which had been kept in a refrigerator, preventing putrifaction with toluene, for a period of approximately one and a half months, was used as the starting material. Every 100 *cc* of the starting material were added with varying amounts of 0.1 *N* acetic acid and distilled water so that a series of samples were prepared in duplicate, throughout which total volume and protein content were kept constant (32.02 *mg* N per 130 *cc*) but pH variant. One of the duplicate was filtered after it had been kept for a night at room temperature (series A); and the other was previously heated for 20 minutes in a boiling water-bath and filtered after it had also been kept for a night at room temperature (series B). The results are given in Table 20,

28) This implies that the heat-effect thus far investigated in this experiment should be of all-or-none nature, and consequently further that the molecular structure being determinant for eumyosin to retain *Kamaboko*-forming ability should be of certain special uniformity.

while the solubilities of heat-treated eumyosin (series B) as well as those of not-heated one (series A) in relation to pH are shown further in Figure 15.

Table 20. Solubility Changes of Eumyosin heated in Acid or Base Solutions.

Number of sample	To every 100 *cc* of the stock sol added (*cc*)		About the filtrate of sample			Filterably dissolved N in per cent of total N (%)	Remarks
	0.1 *N* acetic acid	Distilled water	pH	Extent of turbidity	Nitrogen per 50 *cc* (*mg*)		
Series A: Not-heated.							
1	2.8	27.2	8.3	Trace of haze.	1.640	13.31	Almost perfectly transparent gel was precipitated.
2	3.4	26.5	7.8	Clear.	0.831	6.74	Filtered very slowly.
3	4.8	25.4	7.3	〃	0.481	3.91	Coagulums floated.
4	7.0	23.4	6.1	〃	0.119	0.97	〃 〃
5	8.2	21.8	5.5	〃	0.084	0.69	〃 〃
6	10.0	20.0	5.1	〃	0.219	1.78	
7	16.0	14.0	4.7	〃	3.695	30.00	
8	30.0	0	4.6	Slightly opalescent.	11.596	94.15	Slightly opalescent, with coagulum in a minute quantity.
Series B: Heated for 20 *min* at 100°C.							
1	2.8	27.2	8.3	Hazy.	10.107	82.05	
2	3.4	26.5	7.8	Trace of haze.	4.333	35.15	Filtered only very slowly.
3	4.8	25.4	7.3	Slightly turbid.	2.688	21.82	Filtered somewhat rapidly.
4	7.0	23.0	6.1	Clear.	0.300	2.43	
5	8.2	21.8	5.5	〃	0.195	1.58	
6	10.0	20.0	5.1	〃	0.124	1.01	
7	16.0	14.0	4.7	〃	0.012	0.01	
8	30.0	0	4.6	Opalescently turbid.	11.128	90.35	

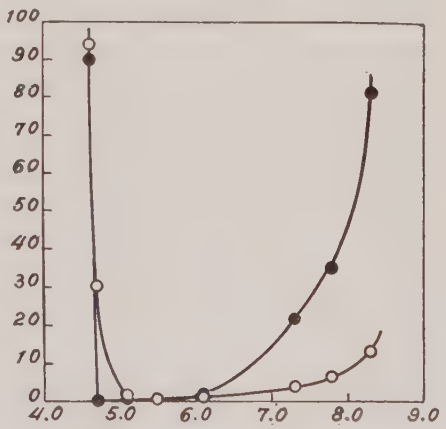

Fig. 15. Solubility-change of eumyosin effected by heat, which had been contained in HCl or NaOH solutions. Abscissa: pH of the medium. Ordinate: Dissolved N in per cent of total N. Open circles: Series A. (Before heating.) Solid circles: Series B. (After heating in a boiling water-bath for 20 *min.*)

These illustrations point to that eumyosin kept in acid or base solutions, when heated at the temperature of boiling water, becomes altered at least in

its solubility as follows: While eumyosin is rendered less soluble on the acid side to the isoionic point, at least in a pH range closely adjacent to it, the protein is rendered more soluble on the basic side to the isoionic point, and the more markedly at the higher pH.

So far as it is assumed that the dissolution of eumyosin should be performed through the dissociation of its micelles, and furthermore, the dissociation, in turn, should be promoted with the increasing net charge on the protein molecules, it must be expected that eumyosin is rendered soluble by heating on the either side of the isoionic point. Although WEBER (1925 b) has early observed on the coagulation temperature of classical myosin such a situation as that the hydrophilicity of the protein seems by no means to be higher on the directly acid side to the isoionic point than that at the isoionic point itself, the above-mentioned recession in solubility observed on the directly acid zone to the isoionic point might rather be due to a modest degradation of cationic eumyosin, which would be accompanied by a shift in the pH of minimum solubility towards a more acid reaction compared with the isoionic reaction of intact eumyosin (*cf.* III, 3, 3), taking into account that the pH of minimum solubility of heat-attacked eumyosin was found at pH 4.7 (*cf.* series B in Table 20).

The spontaneous changes in solubility occurring in somewhat strongly acid or basic sols of eumyosin will be described elsewhere.

III, 3, 2. Effects of Heat on Gelating Ability.

Ionogelation of eumyosin. As often stated, eumyosin suspension forms peculiar coarse colonies of flocculum at a pH of about 8.5, especially in the practical absence of salt. This property characteristic to the fresh preparation of eumyosin seems to be a sufficient factor for the phenomenon of *Kamaboko*-formation. Any eumyosin preparation possessing this property is invariably capable of *Kamaboko*-formation even if the preparation is somewhat different from a fresh one, for instance, in the solubility in neutral salt solutions. Although any explicit interpretation has not yet been given in literatures (*cf.* EDSALL, 1930; FERRY, 1948) as to the origin of this property, the author will call this phenomenon of the formation of peculiar colonies "protonogelation", or more generally "ionogelation" of eumyosin, because it takes place only when the acid-base equilibrium or ionization equilibrium of eumyosin lies within a special range of pH, *i.e.*, of proton activity of the medium.

If a highly concentrated deposit of eumyosin, *e.g.*, such as obtained by centrifugation at the isoelectric point, is added with not too dilute a solution of NaOH little by little until the reaction attains to a pH of about 8.5, it becomes soon remarkably viscous or sticky as if it were made of fresh rice-cake just pounded. The highly viscous paste-like substance here produced, however, loses gradually its fluidity during allowed to stand and sets finally into a semi-transparent *Konnyaku*[28]-like mass of jelly accompanied by a marked elasticity. The phenomenon underlying this series of changes may certainly be the protonogelation. Now, if the *Konnyaku*-like mass of jelly is put into boiling water,

28) *Konnyaku* is a very popular sort of food in Japan, which is prepared from the root of *Hydrosome Rivieri*.

it turns opaquely white in a short time and comes floated to the upper layer of the bath, whilst it falls again to the bottom on cooling. It was actually experienced that the final product obtained on heating the eumyosin jelly which had been prepared thus through protonogelation was essentially the same as the typical *Kamaboko* of a high class in the respect that it was not only excellently elastic but also very nice to the taste excepting that it had not been seasoned differring with normal *Kamaboko*.

Heat-damage on protonogelating ability. In general, eumyosin being dissolved in a dilute solution of HCl or NaOH in the practical absence of salt is not flocculated on heating; in other wards, the coagulation of eumyosin is realized only in such a system as that wherein at least a portion of the protein lies already flocculated. This means, however, neither that the heat-coagulation is realizable only between the ready-made flocculums nor that the ready-made flocculums behave always as coagulable on heating, as it has already been exemplified in the preceding section, III, 3, 1, 3. When flocculated eumyosin is forced to be dissolved sorely by heating, the original ability of protonogelation, or protonogelatability in brief, is often lost permanently, while the loss in protonogelatability, in turn, takes place the more readily at a pH the more distant appart from the isoionic reaction.

A few examples of experiment wherein the effect of the pH of medium upon the thermal damage on protonogelatability was pursued against the duration of heating (at 40°C) are given in Table 21 as well as in Figures 16 a and 16 b. In these experiments, remaining protonogelatabilities were determined quantitatively according to the procedure which will be described in Part III of the present work.

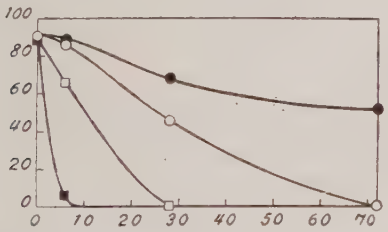

Fig. 16 a. Failure in protonogelatability of eumyosin stored in water at various pH's. Samples of series A. Abscissa: Time of storage in hour at 40°C. Ordinate: Recovered eumyosin in per cent. Open circles: at pH 2.45. Solid circles: at pH 5.5. Open squares: at pH 11.4. Solid squares: at pH 12.1.

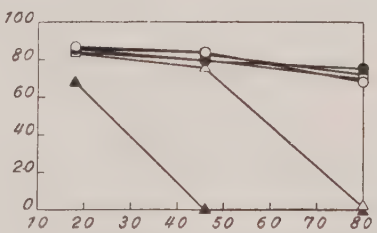

Fig. 16 b. Failure in protonogelatability of eumyosin stored in water at various pH's. Samples of series B. Abscissa: Time of storage in hour at 40°C. Ordinate: Recovered eumyosin in per cent. Open circles: at pH 4.5. Solid circles: at pH 5.5. Open squares: at pH 7.0. Solid squares: at pH 7.5. Open triangles: at pH 8.2. Solid triangles: at pH 9.0.

From these illustrations, it is seen that the rate of the thermal recession in protonogelatability depends upon the pH of medium as well as on the time of heating:

(1) During the storage in warm (40°C), protonogelatabilities recede in general somewhat deliberately in the beginning but vividly in the midway until

the recession slows down again at last.

(2) The rate of the recession is markedly reduced in the isoionic zone (pH 5–6); but the rate at the isoionic reaction is not always reproducible between different preparations of eumyosin.

Table 21. Failure in Protonogelatability of Eumyosin stored in Water at Various pH's. (Temperature of Storage=40°C.)

Period of storage (*hrs*)	Condition of sample		Eumyosin was			Series of sample
	before storage	after storage	changed to be insoluble %	changed to be not-gelatable %	re-covered %	
			at pH 2.45			
1.0	Bulky deposit; clear supernatant.	Bulky deposit; clear supernatant.	−0.8	7.3	91.1	
6		〃 〃	3.3	10.4	86.3	
28		Uniformly turbid liquor; viscous but not elastic.	−0.8	54.5	46.2	Series A (Stored at 40°C since July 9.)
72		Uniformly turbid, non-viscous.	0.3	99.0	0.8	
			at pH 4.5			
18	Uniformly turbid; looked like thin rice-gruel.	Uniformly turbid; looked like thin rice-gruel.	−0.2	13.1	87.0	
46		〃 〃	0.8	14.4	84.3	Series B (Stored at 40°C since July 16.)
80		Voluminous coagulum floated; slightly turbid mother liquor separated.	3.5	28.2	68.3	
			at pH 5.5			
6	Clear supernatant was separated.	Colonies of coagulum floated; mother liquor clear.	0.1	11.0	89.0	
82		Brownish, compact coagulum floated; mother liquor clear.	0.8	31.6	68.2	Series A
72		Orangish, compact coagulum floated; mother liquor clear.	1.4	44.9	45.2	
18	Clear supernatant; greyish white, opaque deposit.	Coagulum floated, clear mother liquor separated.	2.9	10.9	85.9	
46		Rice-cake-like coagulum floated; clear mother liquor separated.	0.2	20.3	79.7	Series B
80		Compact coagulum precipitated; mother liquor clear.	−0.6	25.0	75.4	

Table 21. (continued)

Period of storage (*hrs*)	Condition of sample		Eumyosin was			Series of sample
	before storage	after storage	changed to be		recovered	
			insoluble	not-gelatable		
			%	%	%	
		at pH 7.0				
18	Uniformly turbid; looked like thin rice-gruel.	Very bulky flocculum floated, separating slightly turbid mother liquor.	0.8	15.0	83.7	Series B
46		Bulky rice-cake-like flocculum floated, separating slightly turbid mother liquor.	0.3	19.2	80.8	
80		Compact coagulum floated, separating somewhat strongly turbid mother liquor.	4.8	28.6	70.0	
		at pH 7.5				
18	Uniformly turbid; looked like thin rice-gruel.	Paste-like tactoidal coagulum floated, occupying a major half of the system; small amount of slightly turbid mother liquor separated at the bottom.	−0.4	13.4	85.4	Series B
46		Upper half of the system was strongly turbid; lower half weakly turbid.	1.1	15.9	83.9	
80		Uniformly turbid liquor.	1.5	26.2	73.9	
		at pH 8.2				
18	Uniformly turbid; looked like thin rice-gruel.	Of the total volume of the system, upper 4/5 were heavily turbid; lower 1/5 slightly turbid.	1.5	16.8	83.0	Series B
46		Upper 2/3 volume were strongly turbid; lower 1/3 moderately turbid.	2.1	23.8	75.7	
80		Uniformly turbid lquior.	0.2	98.0	2.0	

Table 21. (continued)

Period of storage (*hrs*)	Condition of sample		Eumyosin was			Series of sample
	before storage	after storage	changed to be		re-covered	
			insoluble	not-gelatable		
			%	%	%	
		at pH 9.0				
18	Uniformly turbid; looked like thin rice-gruel.	Uniformly turbid not-viscous liquor.	0.3	34.1	68.5	Series B
46		Upper 3/4 volume were strongly turbid; lower 1/4 weakly turbid.	0.8	99.1	0.2	
80		Uniformly turbid liquor.	1.2	100.2	−1.5	
		at pH 11.4				
6	Elastic liquor at the beginning; lost the elasticity in a while; semitraesparent.	Uniformly turbid liquor.	0.3	33.1	66.6	Series A
28		〃 〃	−0.2	100.3	−0.2	
72		〃 〃	−1.9	102.1	−0.3	
		at pH 12.1				
0.1	Elastic liquor at the beginning; became not-elastic in a while; semi-transparent.		0.9	10.1	89.0	Series A
6		Uniformly turbid liquor.	0.4	94.2	5.9	
28		〃 〃	0.5	102.6	−2.9	
72		Slightly turbid liquor; tinged orange.	0.8	100.0	−0.8	

(3) Protonogelatability recedes more promptly at basic reactions than at acid reactions to the isoionic zone.

(4) The rate of the recession is the larger at such a pH where the solubility of eumyosin attains to the larger value.

The unique prevalent conclusion arrived at through the present experiments is that the protonogelatability of eumyosin is damaged by heat the more readily, the larger portion of the protein is kept dissolved. This points to that the change in solubility caused by heat is of irreversible nature.

From the conclusion just mentioned, it is further inferred that the extent to which the surfaces of eumyosin particles are exposed directly to the action of hot water is of great consequence. The porocity of the deposit mass of eumyosin in the isoionic zone, however, is expected to vary according to circumstances presumably owing to the possible association between the particles in

successively different levels of fineness, *e.g.*, intermolecular association, intermicellar association, and *etc.* With the case of a highly porous deposit possessing a larger surface exposed to the surrounding water, the heat-action of the medium should effect more rapid destruction of protonogelatability than with the case of a dence deposit. Standing on the wellknown fact that the deposit of classical myosin in distilled water becomes spontaneously less and less soluble, it seems quite natural that some discrepancies are detectable between the results of series A and those of series B: The stability of eumyosin against heat, at one and the same pH of 5.5, was found to be far much greater, in the case of series B than in series A, on which the measurement of remaining protonogelatability started on July 16 and on July 9, respectively, while the same stock of isoionic precipitate having been prepared on the beginning of July was used as the starting material for both the series. The abovementioned discrepancy appears to point out that the storage of eumyosin in the isoionic state is rather effective to protect the protein from the thermal loss in protonogelatability.

It should be noticed that, however, it remains yet uninterpreted up to the present the question, that is, what is the origin at all of the wellknown fact that classical myosin deposit in distilled water becomes spontaneously less and less soluble during the storage. Because this phenomenon seems to have certainly an important connection with the key to the problem of the powder of raw fish, it should be discussed more fully and also given an explanation later on (*cf.* IV, 4, 2) from the standpoint of colloid chemistry.

Hydrogelation of eumyosin. A concentrated sol obtained by liquefying a centrifuged deposit of eumyosin with a small amount of not too dilute a solution of NaOH,[30] when cooled in a refrigerator, sets into a transparent mass of jelly just like a common gelatin sol does on cooling. The jelly produced here, however, is *far much fragile*, resembling agar-agar-jelly, compared with normal *Kamaboko*-jelly. It is liquefied on being brought back to room temperature, but solidifies again on cooling. Such a thermal sol⇄gel transformation of eumyosin at a slightly basic reaction, pH 10 to 11, can be repeated many times until the summation of the period during which the protein kept liquefied, *i.e.*, at room temperature, grows at last so large that the gelating ability becomes abolished.

The large stability of the jelly at a low temperature implies that the temperature coefficient of the velocity constant of the thermal denaturation reaction of eumyosin carrying plenty of net charge might certainly be of some huge value; in other words, the intramolecular disordering of eumyosin (*cf.* IV, 4, 1, Remarks 5) causing the loss of gelating ability may be such a sort of reaction as demands a huge activation energy. On the other hand, the fact that mere fluctuation in temperature effects the reversible sol⇄gel transformation suggests that the transformation is directed merely by the fluctuation in the degree of hydration of the protein. So, the gelation in the present case should be called "hydrogelation" in contrast with "ionogelation" mentioned above.

It is likely that the thickening of the hydration layers enveloping the micelles, which is promoted with the drop in temperature, leads the delicate

30) This sol is suitable to be spun into a filament by using an acid bath.

fibrous micelles of eumyosin into less flexible but more extended ones so as to come into contact with each other, building-up a network of jelly, while the decrease in hydration caused by the rise in temperature reduces the effective thickness of hydrated micelles and leads them back into flexible convoluted ones whose form is statistically more probable than an extended form, thus resulting in the resolution of the network structure of jelly. The experience that the sol⇄gel transformation related to hydrogelation is carried out without accompanying any appreciable time-lag seems to support the abovementioned view of the author.

Although *Kamaboko*-jelly-formation, whether be it accomplished through protonogelation or through salting-in, cannot be considered with indifference to the hydration of eumyosin molecules, which precedes their ionization (*cf.* Kendall and Booge, 1917), it seems likely to be an event brought about among far larger particles than such incipient micelles as considered in the case of hydrogelation. The most plain distinctions between the behaviors of *Kamaboko*-jelly and the jelly produced through hydrogelation is that: while the former is rather tough and hardened on heating, the latter is fragile and liquefied on heating.

III, 3, 3. Disintegration by Acid or Base

As already mentioned, if an aqueous system of eumyosin, whatever pH it may have, is heated for a long period of time, a more or less appreciable portion of eumyosin is affected by heat so as to remain dissolved in the mother liquor when the reaction is subsequently adjusted to pH 5.5 at which the original protein is wholly flocculated. The water soluble proteinous matter produced here should be named by the author provisionally *metaeumyosin.*

Metaeumyosin cannot be precipitated by dialysis against distilled water, but can be readily salted-out with NaCl; its aqueous solution gives no flocculum if heated to boil, exhibits far much lower viscosity compared with a sol of pseudomyosin, and shows no flowbirefringence in contrast with the latter. It is likely that, therefore, metaeumyosin is a sort of derived protein from eumyosin.

Experimental. Table 22 which follows contains the results of an experiment in which the feature of the conversion of eumyosin into metaeumyosin, which is promoted by coexistent HCl or NaOH, especially by the latter, was researched. The observed changes in solubility as well as in viscosity are also plotted against pH of the medium respectively in Figures 17 a and 17 b.

In this experiment, the material for the preparation of samples was obtained by repurification of an eumyosin stock at pH 8.5 which had been reserved in a refrigerator for about three months and used in the form of suspension at a pH of about 5.9. The stock had been kept under the saturation with toluene, and was found to be protected perfectly from putrifaction.

Throughout all the samples belonging to a respective series, total volume and protein content were kept respectively at definite values; but pH was variant in every one of the samples. The samples belonging to series A were prepared as follows: Several sets of the starting-material were prepared by dissolving a part of the repurified eumyosin in an ice-cold NaOH solution so that pH of 12.00 was attained, and subsequently titrated back with various amounts

Table 22. Changes in Solubility and Viscosity revealed by Eumyosin preserved in an HCl or NaOH Solution.

Number of sample	pH of sample at 18°C		Dissolved N in *per cent* of total N	Relative viscosity at 25°C η_R	protein in *gram* per 100 *cc* of sample (C_p)	Intrinsic viscosity $\left(\frac{log\ \eta_R}{C_p}\right)$
	result	method				
			Series A: Control			
12	2.261	Electrometric	5.6	1.025	0.00939	1.14
11	2.875	〃	2.8	1.017	0.00501	1.41
10	4.00	Methyl orange	1.4	1.001	0.00251	——
9	4.30	〃	0.8	1.004	0.00146	——
8	5.29	Methyl red	0.7	1.000	0.00130	——
7	6.73	〃	1.1	1.010	0.00194	—— *
6	7.55	Bromthymol blue	1.6	1.005	0.00291	——
5	7.85	〃	4.0	1.020	0.00857	1.00
4	8.00	〃	9.0	1.031	0.01634	0.81
3	10.833	Electrometric	101.2	1.238	0.1838	0.505
2	11.539	〃	98.5	1.138	0.1788	0.314
1	12.004	〃	99.3	1.098	0.1801	0.281
			Series B			
1	2.423	Electrometric	7.92	1.023	0.0151	0.15
2	3.074	〃	5.88	1.019	0.0112	0.73
3	4.468	〃	1.57	1.017	0.0030	——
4	4.954	〃	1.09	1.006	0.0021	——
5	5.27	Methyl red	1.57	1.024	0.0030	——
6	6.12	〃	1.81	1.003	0.0035	—— *
7	5.88	〃	1.30	1.007	0.0025	——
16	5.256	Electrometric	42.70	1.004	0.0813	0.02
15	5.970	〃	73.30	1.015	0.1392	0.047
14	6.682	〃	98.29	1.024	0.1854	0.056
13	7.649	〃	99.60	1.024	0.1903	0.054
12	9.634	〃	100.05	1.035	0.1947	0.077
11	11.242	〃	101.94	1.041	0.1807	0.097
10	11.915	〃	98.24	1.026	0.1851	0.060
9	12.351	〃	99.04	1.046	0.1873	0.104
8	12.663	〃	101.56	1.032	0.1921	0.071
			Series C			
10	2.441	Electrometric	98.5	1.020	0.1790	0.048
9	3.230	〃	101.6	1.012	0.1374	0.038
8	3.472	〃	100.1	1.018	0.1348	0.050
7	4.041	〃	59.3	1.010	0.1062	0.041
6	4.351	〃	49.8	1.006	0.0893	0.029
5	4.850	〃	48.6	1.003	0.0871	0.016
4	5.972	〃	95.7	1.016	0.1715	0.040
3	6.65	Methyl red	97.5	1.005	0.1719	0.013
2	7.582	Electrometric	100.0	1.024	0.1822	0.057
1	8.764	Phenolphthalein	97.5	1.017	0.1837	0.040

* Calculation of intrinsic viscosity seems to be meaningless in these samples on account of their too low protein concentrations.

of an HCl solution after the basic sol was kept in a refrigerator for a night. In series B, a group of samples, *i.e.*, No. 1–No. 6, were first acidified to pH 2.42 by the addition of HCl and kept for fifteen days in the cold season at the temperature of laboratory without heater; and subsequently they were titrated back with an NaOH solution so that various pH values distributed over a wide range of pH were attained. Another group of samples, *i.e.*, No. 8–No. 16, on the other hand, were previously alkalinized to pH 12.66 and kept side by side with the acidified samples, No. 1–No. 6, mentioned above, and afterwards titrated back with varying amounts of an HCl solution so that they were arranged over a wide range of pH. Only sample No. 7 remained out of connection with such antecedents. Finally, in series C, the samples having been first alkalinized to pH 12.66 were kept at 33°C. for three days in an incubator and subsequently titrated back with an HCl solution to various pH's; however, they smelt of ammonia, though only very faintly, before they were titrated back, suggesting that some chemical decomposition had certainly occurred in them.

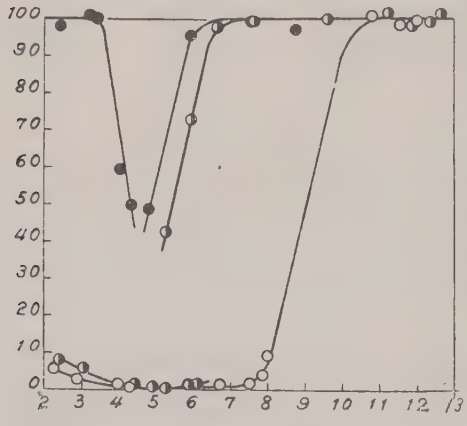

Fig. 17 a. Solubility of eumyosin previously kept in an HCl or NaOH solution (*cf.* the text). Abscissa: Final pH. Ordinate: Dissolved N in per cent of total N. Open circles: Series A. Semisolid circles: Series B. Solid circles: Series C.

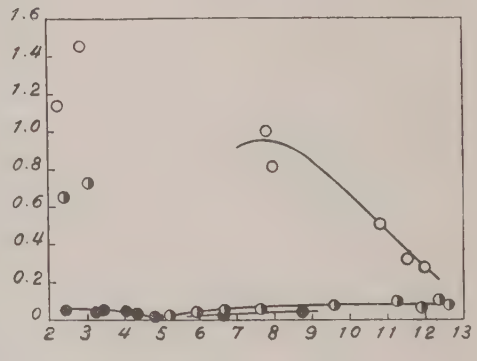

Fig. 17 b. Intrinsic viscosity of eumyosin previously kept in an HCl or NaOH solution (*cf.* the text). Abscissa: Final pH. Ordinate: Intrinsic viscosity at 25°C. Open circles: Series A. Semisolid circles: Series B. Solid circles: Series C.

The measurements were carried out after the unevenness in NaCl content produced by the differential back titration had been corrected by the addition of NaCl and distilled water throughout all the samples. (Final NaCl content was calculated to be 12.8 *millimol*/250 *cc*, *i.e.*, 0.0512 *mol*/*litre*.) The figures of viscosity listed in Table 22 were obtained on the mother liquors, *i.e.*, the filtrates of the samples, while the filtration were carried out after the samples had been allowed to stand for a night so that a virtual equilibrium might be attained. The basic samples were filtered specially in closed vessels so as to be kept from atmospheric CO_2.

It is seen in Figure 17 a that the solubility *vs.* pH relation of the samples in series A appears essentially parallel with that of a typical preparation of

intact eumyosin (*cf.* Figures 4 and 12). The behavior of eumyosin kept in rather a strong HCl solution, *i.e.*, the starting-material for samples No. 1–No. 6 in series B, is approximately the same as that of intact eumyosin excepting that the former becomes a little more insoluble in the acid pH range to the isoionic point of the latter.[31] On the other hand, eumyosin which has been kept dissolved in rather a strongly basic solution of NaOH, *i.e.*, the mother material of samples No. 8–No. 16 in series B, becomes completely changed in solubility; it behaves, namely, as more or less readily soluble than the intact material at least in the basic pH range to the original isoionic point.[32] The precipitate of thus modified eumyosin has a flour-like appearance in opaquely white tinged with brown and looks by no means gelatinous. In this case, the protein becomes quite different to intact eumyosin also as to viscosity, *i.e.*, any appreciable rise in viscosity does not occur on neutralizing the basic sol.

If eumyosin dissolved in a solution of NaOH is kept in warm, *e.g.*, as in series C, it suffers a more serious mutation and becomes so as to remain dissolved more or less completely in the mother liquor even when it is brought to the condition of minimum solubility which is in turn markedly shifted towards a more acid pH compared with the isoionic point of intact eumyosin.

In short, eumyosin dissolved in an NaOH solution loses its protonogelating ability with ease, while the same in an HCl solution does so rather hesitatingly.

The reason why the protonogelatability is abolished by heat, being accelerated by acid or base, should be considered elsewhere after the origin of the protonogelatability itself has been submitted to discussion (*cf.* IV, 4, 1).

III, 3, 4. Spontaneous Coagulation

As already known on classical myosin (Weyl, 1877–8; Edsall, 1930), it has been experienced with eumyosin too that: Whereas fresh precipitate of eumyosin can be readily dispersed in a solution of neutral salt of moderate concentration, the precipitate becomes agglomerated in time into a mass of jelly which, in turn, undergoing syneresis, shrinks spontaneously into such a coagulation as is virtually insoluble in the salt solution as well as in acid solutions, *e.g.*, of HCl or acetic acid, *etc.* Even eumyosin dissolved in a salt solution, forming a sol, if it is of somewhat high concentration, also exhibits, when activated by heat, a tendency similar to that which is revealed by precipitated eumyosin (*cf.* Figures 13 a, b, and c in III, 3, 1).

However, even coagulated eumyosin thus has become difficultly-soluble in

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31) In this connection, it should be remarked that eumyosin thus rendered more difficultly-soluble in HCl solutions visualizes the existence of a minimum in sediment volume also on the acid side to the isoionic point. Standing on this result of observation, the previously mentioned fact that freshly precipitated eumyosin does not exhibit in the acid pH range to the isoionic point any distinct minimum in sediment volume may certainly be ascribed to its too steep increase in solubility in a narrow pH range slightly acid to the isoionic point.

32) This warns us of that not only the temperature but also the period of maintenance of bleached fish in contact with NaOH should be of major significance in the author's procedure for the preparation of eumyosin (*cf.* Part III).

a neutral salt solution as well as in an HCl solution, if it is once dissolved in an ice-cold solution of NaOH and subsequently flocculated again by the addition of acid, resumes not only the property of salting-in but also the distinct protonogelating ability which intact eumyosin has had. If all the operations are carried out at a temperature as low as 0°C, such a cycle as this can be repeated time after time. Consequently, both the spontaneous coagulation of eumyosin deposit and the heat-flocculation of eumyosin suspension may be regarded as *homodrom* irreversible (Pauli and Valko, 1933), but cyclically reversible, phenomena depending essentially on the same mechanism of reaction.

Some experimental data available to elucidate the nature of these phenomena will be given in the following.

III, 3, 4, 1. The cyclic reversibility of heat-coagulation

In this experiment, a weakly basic sol obtained by dissolving a fresh preparation of eumyosin in an ice-cold dilute solution of NaOH was used as the stock solution. Every 100 *cc* of the stock solution were added with various amounts of 0.1 *N* HCl and distilled water so that three duplicated groups of intermediate samples were prepared, every one of which, in turn, consisted further of four members being variant from one another in pH, *i.e.*, approximately 7.0, 6.0, 5.5, and 5.0 respectively by pH. All the samples were kept uniform in total volume (120 *cc*) as well as in nitrogen content (27.08 *mg*/100 *cc*). Every four duplicated members of the intermediate samples were made into the final samples of three sections according to the procedures given below:

(a) Every 100 *cc* of the intermediate samples were added with various amounts of 0.1 *N* NaOH and distilled water so as to arrive equally at pH 7.0 and a definite value in total volume (125 *cc*), then diluted with equal amounts of 1.0 *M* NaCl, and used as the final samples belonging to group a.

(b) Another 100 *cc* of them were put into each one of Erlenmeyek flasks fitted with a cork through which a long glass tube working as a reflux condensor was inserted, They were first heated at 70°C for 80 minutes,[33] and then cooled down rapidly under tap water immediately after the heat-treatment had been completed; then, they were treated in a way just the same as with the case of (a), having resulted in the final samples of group b.

(c) At first, such samples as were quite parallel with the final samples of group b were prepared. They were than added with every 5.0 *cc* of 1.0 *N* NaOH and preserved in a refrigerator for 42 hours so that they become apparently into a state of complete dissolution.[34] Finally, they were brought back again to pH 7.0 with every 5.0 *cc* of 1.0 *N* HCl, and added with 115 *cc* of 1.0 *M* NaCl so as to be used as the final samples of group c.

33) There have been appeared early many publications such as those wherein the coagulation-temperature of myosin is tought to be 50-52°, while the datum seems to be referred to the paper of v. Fürth (1895).

34) During preservation, the samples were stirred up from time to time, but the heat-coagulum formed at pH 7 dissolved only far more hesitatingly compared with coagulums in other samples, suggesting that heat-coagulation has proceeded most intensely at pH 7.

All the final samples were filtered; and the solubilities of heat-coagulated eumyosin, resumable and not-resumable, were estimated with the filtrates, while the solubilities were represented in the percentage of the nitrogen thus dissolved in 0.5 *M* NaCl at pH 7.0 against the total nitrogen of the samples. The results are illustrated in Table 23.

Table 23. Thermal Failure and Cyclic Restoration of Salting-in of Eumyosin.

	Series number of sample	No. 1	No. 2	No. 3	No. 4	Remarks
	pH of sample when heated.	7.0	6.0	5.5	5.0	
N salted-in in 0.5 *M* NaCl	Group a { in *mg*/100 *cc*.....	8.35	8.21	8.16	8.27	} initial
	{ in *per cent* of total N	77.2	75.8	75.4	76.4	
	Group b { in *mg*/100 *cc*	0.05	0.08	-0.02	0.03	} abolished
	{ in *per cent* of total N	0.5	0.7	-0.2	0.3	
	Group c { in *mg*/100 *cc*	5.58	7.81	7.97	8.12	} restored
	{ in *per cent* of total N	60.8	72.1	73.6	75.0	

It is inferred from the data given in the table that the salting-in property of eumyosin in an NaCl solution, even if once abolished, *i.e.*, rendered implicit, through heat-coagulation, is cyclically resumable. As seen in the table, the salting-in property has been recovered only less completely in series No. 1 (at pH 7.0) than in other series so far investigated. But, as it was observed that the heat coagulum formed here also entirely dissolved at least in the ice-cold solution of NaOH even if the dissolution proceeded only very slowly, it is presumed that the short recovery achieved in this case may have originated in some thermal change different from intermicellar heat-coagulation, *i.e.*, in the intramolecular change accelerated by NaOH (*cf.* III, 3, 1, 2; III, 3, 3). Although such concentration in NaCl as low as 0.5 *M*, of course, scarcely corresponds to that of such a solution as that wherein the salting-in of eumyosin attains to a maximum, the reason why the figures representing the solubilities do not attain to 100% even with the case of group a, especially with sample No. 1, seems to be attributable rather to the fact that the eumyosin sol in an NaCl solution behaves in general so that a more or less appreciable portion of it cannot pass through filter paper despite of that it behaves stably against the gravitational force (*cf.* III, 2, 4).

Now, on the other hand, it was verified, on the remainder of the duplicated samples of group c, some aliquot portions of which had been spent in estimating the total-nitrogen content, that they underwent syneresis followed by flocculation (*cf.* Figures 13 a, b, c) when heated in a boiling water. It may be inferred from here that the ability to be salted-in as well as that of heat-coagulation which are characteristic to intact eumyosin in an NaCl solution of moderate concentration are not lost essentially even though the protein is subjected to heat-coagulation if the reaction of environment is kept within a range of pH 5–6.

Furthermore, as a matter of great importance, it was experienced that the eumyosin precipitate recovered from an ice-cold NaOH solution of the heat coagulum resulted in a marshmallow-like substance when it was strained between

dry sheets of filter paper; and the marshmallow-like substance, in turn, was further transformed into a typical mass of *Kamaboko* when heated in hot water. It is evident, therefore, that *Kamaboko-forming ability is* also *cyclically resumable from heat-coagulated eumyosin.*

III, 3, 4, 2. Effect of concentration.

To carry out the sampling in this experiment, four different materials were prepared from a routine deposit of purified eumyosin. They consisted of, respectively, (1) the intact deposit itself, (2) a more concentrated deposit obtained by centrifuging the original (intact) deposit, (3) a marshmallow-like mass obtained by straining the centrifuged deposit after it had been subjected to syneresis between the sheets of *Habutae* laid over the layers of dry filter paper, and (4) a fairly desiccated material obtained by pressing the marshmallow-like substace against a dry porous plate of earthenware.[35] In order to visualize the degrees of concentration of these materials, their relative water content referred to the unit weight of anhydrous matter are listed in the third column of Table 24 a.

Of each one of these four preparations, an calculated amounts (100 *mg* by nitrogen content) was placed in every one of beakers of 100 *cc* capacity, added with distilled water by such a quantity as is respectively calculated from the moisture content given in Table 24 b and further with 250 *cc* of 2 *M* potassium phosphate buffer at pH 7 so that the total water content attained to a definite value (500 *cc*), and subsequently kept under gentle stirring; it was thus intended to shut out the possible error occurring from the difference in the amount of saturating body. A suitable portion of the mother liquor was taken out at intervals; and the nitrogen concentration in the mother liquor (4.00 by ionic strength; 0.67 *M* in phosphate) was estimated from time to time on the filtrates of the portions thus taken out. The results of all the four preparations are collected in Tables 24 a and 24 b.

It is conceivable from Table 24 a that the dissolution of every preparation attains to equilibrium within 20 minutes. At the same time, it should be noticed that the difference in the degree of concentration gives rise to the difference in solubility, *i.e.*, not in dissolution velocity but in equilibrium concentration. This suggests that there prevails a so-called *denaturation* phenomenon among the present preparations of eumyosin. Anyhow, once precipitated eumyosin recedes rapidly in the solubility in a neutral salt solution with the increase in protein concentration, *i.e.*, with the advance of dehydration of the precipitate.

It is inferred from the result of the present experiment that *the solubility measurement on eumyosin*, if it is required, *should be performed in the direction*

35) When salt-free deposit of eumyosin at pH 6.5, which appeared as white semitrasparent paste-like material, was concentrated by centrifugation, it was compacted into a mass of jelly. On further straining the jelly on a dry porous plate, it became in a while highly viscous and malleable. (If necessary, the jelly was able to be extended into a film over the plate and stripped off afterwards from the plate as a sheet of transparent film.) When the jelly was allowed to stand on the plate so as to be concentrated spontaneously, it resulted in a semitransparent *Kamaboko*-like mass provided with a marked elasticity.

leading the protein to flocculation but should not be towards dissolution.

Table 24 a. Failure of Salting-in of Eumyosin caused by Concentration.

Number of sample	Method of preparation of concentrated sample	Volume occupied by 100 *mg* N in sample (*cc*)[36]	N filterably salted-in in 0.67 *M* K-phosphate buffer at pH 7 (*mg*/100 *cc*) after				
			10 *min*	20 *min*	40 *min*	80 *min*	160 *min*
1	Precipitated under the gravitational force.	500	7.03	7.67	7.67	7.66	7.67
2	Centrifuged.	43	5.21	5.20	5.21	5.21	5.18
3	The centrifuged deposit was submitted to syneresis on dry filter paper.	4	1.35	1.32	1.33	1.31	1.35
4	A part of sample No. 3 was pressed against dry porous plate.	1	0.21	0.20	0.21	0.20	——

Table 24 b. Failure of Salting-in of Eumyosin caused by Concentration. (on the very samples in Table 24 a)

Number of sample	Degree of concentration			Degree of salting-in in 0.67 *M* K-phosphate at pH 7	
	Per 100 *g* of sample		Volume occupied by unit *gram* of N (*cc*)	N per 100 *cc* of the filtrate (*mg*)	Filtered N (%)
	Moisture (*g*)	N (*mg*)			
1	99.87	19.9	5000	7.67	57.5
2	98.50	231.7	430	5.21	39.1
3	90.38	1480	40	1.33	10.0
4	55.21	6890	10	0.21	1.6

III, 3, 4, 3. Effect of temperature

In this experiment, three test materials were prepared as follows from one and the same stock of eumyosin deposit that was used in the above experiment: A portion of the stock was first of all concentrated into a mass of jelly by centrifugation. (1) A first division of the jelly mass was dehydrated with porous plate and subsequently dried up over P_2O_5 in vaccum at room temperature. (2) A second division was dried up in vaccum at 66°C, the boiling point of methylalcohol under the atmospheric pressure. (3) A third was dried up at 100°C in vaccum. These three kinds of dried material were respectively crushed in a mortar into powders finer than 100 *mesh* and used as test material.

The degrees of difficult-solubility of these materials in a neutral salt solution were determined as follows: Every calculated amount of these powders

36) The bulkiness of each preparation (sample) was estimated on the basis of the apparent specific volume and the nitrogen content measured. For sample No. 1, the directly observed sediment-volume was adopted as the apparent specific volume, while for other samples, apparent specific volumes were determined on the basis of the rise of the meniscus of toluene contained in a small cylinder, which was realized when a known amount of the preparation was submerged in the toluene.

corresponding to 300 *mg* by anyhydrous matter, was put into every one of calibrated glass tubes with a definite diameter (15 *mm*), and every 20 *cc* of 0.6 *M* potassium phosphate buffer at pH 7.0 were added. The tubes were allowed to stand for a night after they were stirred up from time to time, and the apparent volumes of sediment layer were read off in order to find the relative extent of swelling for each sample. Then, every 10 *cc* of the supernatant liquor were put into every one of separate tubes the same in type as those previously used, to which every 2 *cc* of 20% solution of sulfosalicylic acid were added so that there took place virtually stable turbidity due to finely dispersed protein flocculums. Three tubes containing the turbid suspensions were kept stand side by side; and suitable amounts of their contents were put away with a pipette from excessively turbid suspensions until they became so as to appear identical with the suspension of least turbidity on looking downward through the suspension layers illuminated from side. The volumes of the suspensions thus remained in the tubes were adopted to express the relative difficult-solubility of the test materials assuming that the turbidity stands in a reciprocal ratio against the difficult-solubility. The result shown in Table 25 points to that eumyosin becomes the more difficultly-soluble when desiccated at the higher temperature.

Table 25. Relative Failure of Salting-in of Eumyosin desiccated at Various Temperatures.

Number of sample	Temperature of desiccation (°C)	Relative sediment volume in 0.6 *M* K-phosphate	Relative extent of difficult-solubility
1	14 (Room temp.)	2.75	1.00
2	66	2.5	1.85
3	100	1.0	2.7

On the difficult-solubility acquired by eumyosin when the protein is heated in aqueous solutions, the descriptions in III, 3, 1 should be referred to.

III, 3, 4, 4. Effect of initial pH

In this experiment, it is inquired into the relation between the initial pH of eumyosin precipitate from which its dehydration is started and the extent of the difficult-solubility arrived at by drying. The samples were prepared as follows: Another portion of the stock of eumyosin precipitate employed in the preceding experiments was subdivided further into several portions, every one of which was respectively brought to various pH's such as those whereat these portions were kept substantially flocculated (pH 5–8). These portions were first strained by centrifugation, then dehydrated by pressing against a porous plate, dried up in a desiccator containing $CaCl_2$, and finally ground up into powders finer than 100 *mesh*. The relative difficult-solubility pertaining to every one of these powders was estimated in just the same way as with the case of the previous emperiment, III, 3, 4, 3. The results are given in Table 26.

As it is noticed in the table, the effect of concentration whose extent is represented in the relative content of anhydrous matter may cause a little

confusion in the present case against inferring the pH-effect on the degree of difficult-solubility, because the former is not kept invariant throughout whole the samples. In samples No. 2–No. 7, however, relative difficult-solubility becomes more and more salient with the rise in initial pH, whereas the degree of concentration decreases steadily against the pH rise; so, it may be regarded, at least for these samples, that the effect of pH increase are predominating over the effect of decreasing concentration. A similar view seems also to hold for the relation between samples No. 1 and No. 2. In short, it is understandable that *the difficult-solubility of eumyosin attained on dehydration is kept minimal when the protein has been initially at the isoionic point.* This is a result quite unexpected, for the protein is most readily coagulated at its isoionic point, and, moreover, it has been tought in general that the coagulation of protein is an implication of its denaturation.

Table 26. Relative Failure of Salting-in of Eumyosin desiccated at Various pH's (at Room Temperature).

Number of sample	pH before desiccation	Degree of desiccation represented in anhydrous matter in *per cent* of dry sample	Apparent sediment velume (*cc*)	Volume of supernatant corresponding to equivalent turbidity (*cc*)	Relative extent of difficult-solubility
1	5.0	87.2	2.2	7.5	1.06
2	**5.5**	**91.5**	**2.6**	**7.2**	**1.00**
3	6.0	90.3	2.5	7.5	1.05
4	6.5	90.8	2.2	7.9	1.10
5	7.0	89.7	1.7	8.5	1.18
6	7.5	89.0	1.5	9.6	1.33
7	8.0	87.8	1.2	12.0	1.67

Remarks 3. From the fact that the difficult-solubility of eumyosin effected by dehydration as well as the dehydration itself do not attain to any optimum when pH 8.5 is approached, it is inferred once more that the protonogelation of eumyosin at pH 8.5 is by no means the indication of a minimum in hydrophilicity; in other words, the protonogelation played by precipitated eumyosin seems *not to be* such a sort of phenomenon as effected by its recession in hydrophilicity. (*cf.* also III, 2, 8).

Remarks 4. The fact that the desiccated eumyosin in III, 3, 4, 2; III, 3, 4, 3; and III, 3, 4, 4 retained such portions, even if in minute quantities, as went dissolved into moderately concentrated solution of neutral salt implies that its difficult-solubility is not directly originated in dehydration itself, but is caused by some change in the molecular state, which is quite different from dehydration but is prone to procession when the protein is dehydrated.

Indeed, the *Kamaboko*-forming ability of eumyosin, under certain circumstances, is not lost even if the protein is completely dried up: For example, an isoionic precipitate of eumyosin was first washed thoroughly with absolute alcohol, and then the residual alcohol was washed off with ether. The white pulpy dry matter thus obtained was proved to retain a marked *Kamaboko*-forming ability at least for several days after having been prepared; namely, when it was previously swollen up in alcohol, and subsequently the alcohol was washed off

with water, the residual substance was converted, on the addition of table salt, into a highly sticky paste, and the paste, in turn, set into an elastic mass of jelly which gave rise to a typical mass of *Kamaboko* on steaming.[37]

37) The fact that the *Kamaboko*-forming ability of eumyosin is not abolished by the treament with alcohol and ether points to that SZENT-GYÖRGYI's myosin, like pseudomyosin of the present author, bears no direct relation to *Kamaboko*-forming ability, for SZENT-GYÖRGYI (1947) says that his myosin is completely denatured by acids or anhydrous solvents.

IV. COLLOID CHEMICAL INTERPRETATIONS OF *KAMABOKO*-FORMATION

IV, 1. Introduction

Referring to the literature, studies on the properties of myosin, which were commenced first by KÜHNE (1859) about a century ago, steady progress as they have made with the advances in physiological and biological chemistry, physical and colloid chemistry, and also in histological techniques, perhaps owing to the vast toilsomeness pertaining to the classical method of its preparation, seem to have been apt to be behind the studies on the other, especially crystallizable proteins. Following the well-known classical publications of detailed works by DANILEWSKY (1881, 1882), HALLIBURTON (1887), v. FÜRTH (1895, 1900, 1919), BOTTAZZI and QUAGLIARIELLO (1912), and others, there have begun to appear systematically the leading works of WEBER and his coworkers (1921–). In about the same decade, the famous works of EDSALL and his coworkers (1930–), in an optical and rheological aspect have been published, while, coming after them, the intensive X-ray studies by ASTBURY and his coworkers (1934–) have achieved brilliant successes in visualizing the intramolecular structure of the protein. It should not also be overlooked the profound contributions made by BATE-SMITH (1930–) to the knowledge on myosin as the determinant of the physical properties of meat. It seems likely that the foundation of the modern physiological chemistry of myosin has already germinated in that decade for the amazing jumps of today (*cf.* ENGELHARDT, 1946; SZENT-GYÖRGYI, 1947). Since the closure of World War II during which the author had been shut out from any more acquaintances with the works having progressed in this field a number of wonderful achivements in the chemistry of myosin including the brilliant successes in the estimation of amino acids on the one hand (*cf.* BAILEY, 1948), and such in the techniques related to electron microscopy (*cf.* WYCKOFF, 1951) on the other hand, have been developed before us. Although an excellent review on the nature of several protein gels has recently been published (FERRY, 1948) and the phenomenon of *Kamaboko*-formation has been taken up again as one of the problems to be attacked (*cf.* TAKAHASHI, MATSUMOTO, and ARAI, 1951; MATSUMOTO, and ARAI, 1951; MIGITA and OKADA, 1952), the mechanism of *Kamaboko*-jelly formation has not yet been given any satisfactory explanation in the light of colloid chemistry of protein. So, the author wishes in the present chapter to give more particularized interpretations of this phenomenon, standing on the basis of the data already described in this paper as well as referring to some foreign works available.

IV, 2. Molecular and Micellar Features of Eumyosin

The form of an eumyosin preparation in which the protein is actually handled is not, of course, a single molecule but always a bulk of system throughout which numberless molecules of the protein are distributed, standing more or less apart from each other. The behavior of such a system should be a function of that of individual molecules, but the functional form may vary ac-

cording to circumstances; namely, the behavior of the system should also depend on the interaction of the molecules involved. From such a point of view, it seems necessary for discussing the affair to take into account not only the molecular state of eumyosin but also the possible feature of micelles composed of the molecules.

Although the studies on the nature of micelle had made a remarkable advance before the war, because it was rather a recent issue that the structure of protein molecule was disclosed to some satisfactory extent (*cf.* COHN and EDSALL, 1943; ASTBURY, 1944; PAULING and COREY, 1951), the effect of molecular structure upon the feature of micelle seems to have yet scarcely been treated in the field of protein chemistry. Under the circumstances, the following discussion on the molecular and micellar features of eumyosin may be rather speculative, though the author thinks it by no means to be quite unavailing.

IV, 2, 1. The Feature of a Singular Molecule

It seems somewhat doubtful whether eumyosin can at all exist stably as isolated molecules (*cf.* III, 3, 1, 2); and the protein is expected to exist usually forming micelles, referring to the electron-microscopic study on myosin carried out by ARDENNE and WEBER (1941). However, since the feature of micelles is expected to depend on that of the constituent molecules, the feature of a singular molecule of eumyosin, be imaginary as it may, should be considered at first.

In the light of knowledges on myosin (not in SZENT-GYÖRGYI's but in the classical definition) (*cf.* BAILEY, 1944), it is inferred that a single molecule of eumyosin is also a sort of polypeptide chain. In order to visualize its feature, however, three factors of essential importance must be taken account of: (1) The existence of cystine bridges which combine at least two polypeptide chains together into a single molecule. (2) The frequent occurrence of ionogenic side chains of various species on the molecule. (3) The remarkable extensibility attaining to as large as 100%. Taking into account furthermore that these side chains are not permitted from the stereochemical point of view to approach each other more closely than a certain limit, a probable example of schema such as shown in Figures 18a and 18b might be given as the molecular configuration of eumyosin. In these figures detailed types of helical turns such as proposed by PAULING and COREY (1951), are abridged to be drawn; but an elliptic type of coiling is assumed considering that cystine bridges would be lying obliquely to the backbone of each polypeptide chain, on account of the distinction in species of the atoms adjacent to α-carbon of cystine, while these atoms, in turn, are assumed to stand in such a manner as resembles the arrangement of trans-type. Furthermore, referring to the independency of 9.85 Å spacing in the X-ray diagram of myosin to its $\alpha \rightleftarrows \beta$ transformation (*cf.* ASTBURY and DICKINSON, 1940), the cystine bridges, the length of which coincides with the said spacing, are assumed to be situated constituting the shortest connections between the two flexible helical peptides which, in turn, stand in parallel to each other. On the other hand, side chains are assumed to be distributed on the surface of the elliptical cylinder formed by the helix, projecting themselves outwards in perpendicular to the axis of the helix, but not towards the neighboring helix in

the same molecule due to the steric hindrance which acts as a stopper against an excessive elongation or contraction of the helix.

The configuration of a single molecule of eumyosin assumed above gives rise to an idea of a three-dimensionally anisotropic field of molecular forces prevailing around the molecule, by which three-dimensionally anisotropic micelles may be built up. The said molecular forces, despite of their three-dimensional anisotropy in respect of intensity, are of dual nature, *i.e.*, COULOMB forces and VAN DER WAALS forces; and the former, whether they are attractive or repulsive, are exerted by the imaginary cylindrical surface of the helix, while the latter are prevailing all over the molecule if not uniformly. The said dual nature in molecular forces constitutes one of the fundamental idea of the present author for his interpretation of ionogelation, *i.e.*, *Kamaboko*-jelly formation of eumyosin.

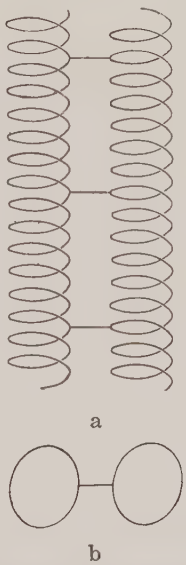

Fig. 18. A schematic representation of the feature of a single molecule of eumyosin.
a. Molecular chain of eumyosin.
b. Cross sectional view of eumyosin molecule.

IV, 2, 2. The Feature of a Singular Micelle

On the basis of MEYER'S (1929) view upon the molecular configuration of protein chains, which is dependent on the pH of environment, it is expected that eumyosin molecules dispersed in a strongly basic solution assume an extremely extended form, for the net charge carried by them should attain to a maximum in such a solution. Accordingly, it might be also expected that eumyosin sols would exhibit a maximal intrinsic viscosity at a strongly basic reaction in so far as the protein is not disintegrated by alkali. However, this is not the case. Namely, in spite of that we can prepare *Kamaboko*-formable eumyosin from an ice-cold sol of the protein dissolved in an NaOH solution whose reaction is strongly basic, the intrinsic viscosity of such a sol is far much lower than that of a faintly basic one as seen in III, 2, 1. It is conceivable, therefore, that eumyosin, in the vicinity of the neutrality of water, exists in a state of molecular aggregate or micelle with an extremely elongated configuration, whereas it is supposed to be dissociated into singular molecules at a strongly basic reaction.

Since the surfaces of the helical peptide chains constituting eumyosin molecules are inferred from the results of titration given in III, 2, 3 to be furnished up to about 50% with ionogenic side chains, the micellar formation of eumyosin is expected to be influenced profoundly by the ionic condition of the envirenment.

In strongly acid or basic solutions, eumyosin will not form micelles, since it acquires dominant net charge which gives rise to a strong intermolecular repulsion. With decreasing acidity or basicity of the medium, the net charge also decreases, leading to the withdrawal in the intermolecular repulsion; this may facilitate the macromolecules of eumyosin to be held aggregated by LONDON-VAN DER WAALS attraction force which is pointed out by VERWEY (1950) to be the

cohesion force in hydrophobic colloids.

Because the potential energy between interacting molecules is expected according to De Boer (1936) to be diminished the more effectively when the larger number of atoms belonging to these molecules come into contact with each other, the molecular configuration of eumyosin considered in IV, 2, 1 suggests that its molecules cohere laterally when aggregated into a micelle. However, since the markedly elongated shape and the flexibility of the molecules renders the chance of their full length coherence to be far much smaller than that of their partial length coherence, their actual aggregation may give rise eventually to extremely long fibrous micelles or fibrils. Thus, the more elongated configuration have assumed the starting molecules, the longer fibrils will be formed through their aggregation. It might be considered, therefore, that the longer micelles are to be formed at the higher acidity or basicity; but, in reality, the intermolecular repulsion due to net charge, being enhanced simultaneously, will, as previously mentioned, prevent the molecules from cohering into micelles at such extreme reactions. Accordingly, the length assumed by a single micelle of eumyosin attains to maximum values at some intermediate pH's which are inferred from the result of viscosity measurements (*cf.* III, 2, 1) to be situated respectively at some pH 8.5 and, quite implicit as it is, at a pH slightly acid to the isoionic point.

At the isoionic point, pH 5.5, eumyosin will acquire a nearly maximal total charge, for, at this pH, all the basic groups and almost all the acid groups on its molecule may be ionized into cation and anion, respectively, as presumed from the ionization constants of its constituent amino acids. Since nearly half of these amino acid residues equip the protein molecule with protolytic groups (*cf.* III, 2, 3), the situation just mentioned will cause the molecule not only to assume a highly folded-up, rather a shortened configuration, as suggested by Meyer (1929), but also to gain a considerable amount of hydration water. Thus, at the isoionic point, the complete recantation in net charge may favor the agglomeration of molecules on the one hand, while the developed hydration due to the dominant total charge may prevent their agglomeration on the other hand. It is expected therefore that, despite of the fact that eumyosin is readily flocculated at the isoionic point, its molecules stand bundled rather loosely within the flocculum. Moreover, on account of their short configuration, the molecules will cohere with one another readily at full length, yielding relatively thick and short micelles. Such a point of view seems to accord with the experimental result that eumyosin can be centrifuged into by far the most compact deposit at the isoionic point compared with at any other pH, whereas the deposit still contains a large amount of water (*cf.* III, 2, 1).

In a pH range of medial basicity, *i.e.*, pH 10 to pH 12, and also in that of medial acidity, *i.e.* pH 4 to 3, where the intermolecular repulsion due to net charge may be fairly strong if not so strong as entirely prevents the molecular association into micelles, the molecules may be kept in contact with each other only when a sufficiently large number of atoms belonging to them have come into contact, for, if only a small number of such atoms exist in contact, they will not be able to hold the molecules whithin a micelle against thermal

agitation. This situation will result in that only not too long fibrils are permitted to be formed such as can exhibit only medial intrinsic viscosities as found actually in the experiments in this connection (*cf.* III, 2, 1).

IV, 3, Ionogelation of Eumyosin

Over ten years ago, the present author reported some experimental results on the gelation of fish flesh; but as he had not at that time arrived at a satisfactory explanation with regard to the mechanism underlying the phenomenon, he assigned the paper as a preliminary report (HIRANO, 1939 b). The centre of the question to be answered is that: Why eumyosin once peptized into a sol by the addition of NaCl transforms spontaneously again into a solid? Although in Part I of the present work (*cf.* foot note 17) a very brief comment on the phenomenon has been given, a more approvable detailed explanation will be given in this section.

IV, 3, 1. The Protonogelation occurring on the Neutralization of NaOH-Eumyosin sol.

The intrinsic viscosity of eumyosin dissolved in a strongly basic solution of NaOH readily falls below some 0.2 unless the sol is kept at a temperature as low as possible, suggesting that eumyosin molecules are very prone to irreversible mutation by heat (*cf.* III, 3, 3). The protonogelation ability of the protein, however, is always resumed in so far as the intrinsic viscosity remains at a value not less than some 0.2. This critical value of intrinsic viscosity, 0.2, corresponds to about 30 by "viscosity increment," ν, instructed by COHN and EDSALL (1943) and hence some 17 by axial ratio, a/b, if an elongated ellipsoidal shapes is assumed for the protein particle, for which a and b are assigned respectively as the magnitude of the semi-axis of revolution of the ellipsoid and that of the equatorial semiaxis.[38]

When a strongly basic sol of eumyosin prepared freshly in an ice-cold solution of NaOH is neutralized by the addition of HCl to a pH of about 8–9, there occurs in the beginning no flocculation of the protein; but the system becomes more and more viscous and at last a portion of the dissolved protein is flocculated almost abruptly forming coarse colonies. This remarkable rise in viscosity seems to indicate that the molecular dispersoids are associated into micelles with a highly asymmetrical configuration, which the author calls primary fibrils for convenience sake. (The mode of aggregation of eumyosin molecules into a primary fibril has already been discussed in IV, 2, 2.)

After primary fibrils have grown up in length to a certain limit, some of the free molecules coming into contact with such fibrils will be able to deposit on the latter at full length, giving rise to lateral growth of the fibrils, while some other free molecules or primary fibrils are attached at partial length as

38) It should be noted that the present author employs in this paper as "intrinsic viscosity" an expression $log\ \eta_R/C_p$ which corresponds to an approximation with the specific viscosity, $\eta_{sp}=\eta_R-1$, of a visional concentrated sol containing unit gram of eumyosin per 100 *cc* of the sol, assuming that ARRHENIUS' equation for viscosity holds throughout the concentration range of $0-C_p\%$ in respect of the protein.

before so that the fibrils under consideration continue their longitudinal growth too.[39] The fibrils which have thus grown beyond the critical length will be called secondary fibrils.

Now that the formation of secondary fibrils should considerably enlarge the chance by which free molecules and/or primary fibrils come into contact with secondary fibrils wriggling about with thermal agitation, once formed secondary fibrils will become autocatalytically so large in length* that such a state is eventually arrived at in which a considerable number of their individuals are quite ready to come into contact respectively with a number of other individuals when their segments happen to acquire a sufficient kinetic energy to jump over the potential hill which is preventing them from colliding with one another.[40] When the majority of secondary fibrils thus have once been fixed as the skeletons of a framework, they cease suddenly to wriggle about, effecting simultaneously a substantial decrease in the chance of further condensation of free molecules or primary fibrils on them; thus, there often remains an appreciable amount of free dispersoid in the bulk of the system after its gelation has apparently come to an end.

On the other hand, it is considered that the stability of the contact once formed between secondary fibrils depends not only upon the magnitude of the interacting segments of these fibrils but also upon the extent of salience of the potential barrier which must have been jumped over by these segments when they have acquired a sufficient kinetic energy to come into contact with each other. Accordingly, the peptizability of an eumyosin jelly is to depend not only upon the ionic condition of medium but also on the length of the time duration for which the jelly has been kept as such.

Since secondary fibrils are considered to grow in some autocatalytical

39) With the case of anisotropic molecules such as presumed above for those of eumysin, their association into fibrils would not be able to be performed in the way proposed by REES (1951), for he implies the amorphousness, or at least the isotropicity, of the particles to be aggregated.

40) Consider a sol being full of enormously-elongated flexible dispersoids. When the segments of such fibrilar dispersoids collide with one another here and there throughout the bulk of the sol, the chance by which some of the collisions occurring in an elemental time interval happen to be carried out upon one and the same fibril will be appreciable. Hence, the chance by which two or more fibrils collide with another fibril in an elemental time will be large enough to make the collisions of fibrils to proceed apparently as a reaction of higher order over third in so far as the system is sufficiently concentrated with free fibrils, while their multiple collisions of the said type may give rise to outstandingly-bulky colonies which function to solidify the whole system apparently into an elastic mass of jelly.

Such an idea as above mentioned might possibly account for the experimental result reported in the author's preliminary paper (HIRANO, 1929) that the gelation of fish paste was found to be a third order reaction: but it remains, of course, another problem whether the assumption made in that paper in order to calculate the reaction order, which postulates that the gelation can be detected first when a certain definite portion of the paste has completed the reaction, is applicable or not.

* The growth in length of flexible fibrils will effect the multiplication of active segments.

manner as mentioned above, it is also predicted that: If not a sufficiently concentrated basic sol of eumyosin is neutralized to pH 8.5 or so, some appreciable portion of the protein will generally be left in the mother liquor after early formed large colonies have promptly settled down, effecting a substantial decrease in the protein concentration in mother liquor. The more diluted the original system, the larger portion of eumyosin will remain despersed in the mother liquor. Thus, the system, while kept standing quietly for a long period, becomes such one as is composed of three distinct layers as shown schematically in Figure 19.

It was experienced, with such a system as is sketched in Figure 19, that the top one of the three layers contained practically no protein; the middle one consisted of a transparent very feeble mass of jelly; and the bottom layer was a bluish white dense mass of jelly. It is likely that the secondary fibrils which were barely formed in a long period of time in the mother liquor whose protein content had already been reduced substantially thus formed a very loose network of jelly having constituted the middle layer.

The proportion of eumyosin precipitated into a compact deposit will thus depend not only upon the total concentration but also presumbly upon the way of agitating the system, the rate of the addition of neutralizing agents, and *etc.*

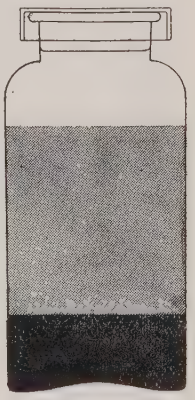

Fig. 19. Double-layered precipitate of eumyosin.

IV. 3, 2. The Protonogelation occurring on the Back-Neutralization of Isoionic Precipitate

If eumyosin is beforehand precipitated completely at the isoionic point, and the deposit is afterwards neutralized back up to a pH of about 8.5 by the addition of a dilute solution of NaOH, a far larger portion compared with the case of IV, 3, 1, *e.g.*, even up to 100 per cent of total eumyosin undergoes protonogelation, setting into a dense mass of jelly.

When isoionic deposit of eumyosin, whose constituent particles may be configured rather short as mentioned in IV, 2, 2, is brought to a pH range of about 6.5—7.0 by the addition of NaOH, its constituent molecules will acquire some net charge and become electrokinetically stabilized, for some of the imidazolium and phenolic-hydroxyl groups on these molecules might possibly become disionized and ionized, respectively. As actually observed, eumyosin deposit at this pH behaves as somewhat fluid. If a dilute system containing such deposit is once stirred up and subsequently allowed to stand, the flocculums sedimentate at a fairly small rate (*cf.* III, 2, 7), pointing to their appreciable fineness and hydrophilicity. On the other hand, eumyosin at pH 6.5—7.0 can be centrifuged into a deposit mass nearly as compact as such centrifuged at the isoionic point, suggesting that the protein particles are configured still short there (*cf.* III, 2, 8). Judging from these results of observation, the increase in net charge in the pH range of 5.5 to 7.0 seems to be performed mainly by new ionization of phenolic-hydroxyl groups but scarcely by disionization of imidazolium groups, *i. e.*, without diminution in total charge.

However, when the deposit is further neutralized to a pH of about 7.5, the ionization of imidazolium groups will presumably be suppressed materially and the eumyosin particles will be imposed substantially with net charge so as to assume a configuration of marked elongation. This view seems to be supported by the experimental result that eumyosin is precipitated only into a highly voluminous deposit layer at pH 7.5 even under a strong centrifugal force (*cf.* III, 2, 8).

Next, when the pH of the environment is raised further back up to about 8.5, all the phenolic-hydroxyl groups on the molecule are supposed to be ionized (negatively), while all the imidazolium groups as well as an appreciable portion of α-ammonium groups will be deprived of their (positive) charge; these will give rise to a further increase in net charge at the expense of total charge, leading the protein into a less hydrated state and also promoting its fibrils so as to attain the same feature at pH 8.5 as that stated in IV, 3, 1. Thus, the fibrils will become linked together in essentially the same way as that discussed in IV, 3, 1 into a spatial network solidifying whole the precipitate into an elastic mass of jelly.[41] A considerable discrepancy, however, is found usually between the result attained in the present case and that in IV, 3, 1 as regards the proportion of precipitated eumyosin to total protein; namely, whereas enmyosin is only partially precipitated when its basic sol is directly neutralized to pH 8.5, almost all the protein is precipitated when the sol is previously brought to the isoionic point and subsequently back-neutralized to the same pH. This may be understood on the idea that eumyosin particles, whether or not they are molecules, primary fibrils, or secondary fibrils, being entirely precipitated at the isoionic point, are piled together electrostatically into a compact layer of deposit so that an extensive connection is gradually developed between them through their thermal vibration, thus bringing all the protein particles eventually into the state of secondary fibrils. The significance of such previous precipitation at the isoionic point followed by backneutralization has been utilized in the procedure to isolate eumyosin (*cf.* Part I, especially II, 3) and will be applied also to the recovery estimation of eumyosin (*cf.* Part III).

The fact that protonogelation occurs most conspicuously at pH 8.5 suggests that fibrous configuration of eumyosin particles may be favored to the utmost at this pH. The formation of a second minimum in sediment volume at the same pH (*cf.* III, 2, 7 and III, 2, 8) seems possibly to be ascribed to the syneresis of eumyosin precipitate, which will be discussed in IV, 4, 2.

IV, 3, 3. Ionogelation caused by NaCl-addition

It is wellknown on the one hand that sodium salts of organic acids or chlorides of organic amines in general are more strongly ionized and more soluble in water than the corresponding free acids or amines respectively, while it is also

41) It should be noticed that the pasty deposit of isoionic eumyosin does neither immediately nor continuously as a whole turn into a mass of jelly even when brought back to pH 8.5 at once; but, in fact, it disperses first into a viscous suspension, and then the fine dispersoids thus once formed clot afterwards suddenly into large colonies of flocculum.

known on the other hand according to Pfeiffer and his colaborators (1923) that amino acids and peptides can interact with salts so as to form amphisalts in which the cation and anion of the salts are combined respectively with the carboxyl groups and amino groups of the amino acids and peptides. Accordingly, it may be inferred that the salting-in of eumyosin by NaCl is attributed to the increase in electrolytic dissociation caused by the amphisalt formation between the protein and the salt.

Since it has early been pointed out by Kendall and Booge (1917) that electrolytic dissociation is preceded by the combination between solute and solvent, eumyosin particles are expected to become more or less strongly enveloped by hydration water when salted-in.* Various kinds of saltbridges, *i. e.*, intramolecular, intermolecular, and intermicellar saltbridges, which would have probably been established within a deposit mass of eumyosin, will thus be released by the action of NaCl when the salt is added. This will further result in the formation of a potential barrier consisting of the layer of hydration water between the particles approaching one another, for their mutual approach will cause a number of water molecules to be compacted additionally. The somewhat elongated fibrilar particles here formed will be rearranged in process of time into a bulky network of jelly so as to decrease the potential of the system, while their segments surmount the potential barrier little by little and come into contact with one another by means of London-van der Waals attraction forces.

However, as it is considered that NaCl cannot confer net charge upon eumyosin, the elongation of its fibrils in this case is not assisted by the intramolecular repulsion due to net charge such as produced when the protein is treated with NaOH. It may be expected therefore that in the case of ionogelation effected by the addition of NaCl, eumyosin jelly is built-up with fibrils not so extremely extended as those in protonogelation; and accordingly it is also expected that the ionogelation by salt can be realized preferably when we start from rather a concentrated suspension or deposit of eumyosin, or else the typical salting-in will result.** Indeed, if we start from rather a dilute deposit of the protein, *e. g.*, an eumyosin jelly formed at pH 8.5, the addition of NaCl will result in liquefaction rather than any further solidification of the jelly just like the case of the thixotropic myosin gel reported by Edsall (1930). The fact eumyosin sols prepared in NaCl solutions appear rather strongly opalescent also implies that the salt does not effect any substantial dissociation of their constituent fibrils.

As is the case with protonogelation, also when a concentrated NaCl-eumyosin sol undergoes ionogelation, the interparticlar linkages being established are considered to be of secondary valence for the resulted gel behaves distinctly as a sort of thixotropic gel (*cf.* Hirano, 1939) though it might eventually undergo syneresis followed by flocculation in a long period of time (*cf.* III, 3, 1, 1).

After all, it may be concluded that the essential reaction responsible for

* That the hydration of protein is really increased when mixed with neutral salt solutions at proper concentrations has been pointed out on serumalbumin dilatometrically by Weber and Nachmannsohn (1929).

** The shortening of fibrils may result in the recesson in the activity, *i, e.*, the effective concentration of reacting segments.

the ionogelation of eumyosin precipitate is not other than the amphisalt formation between the protein and NaCl added, which alters intermicellar salt-linkages into bridgings of VAN DER WAALS-LONDON forces. The reason why the precipitate is peptized under one and the same condition wherein the protein undergoes coagulation may be interpreted from the reasonable conception that a substantial difference in the rate of procession can exist between the peptization and the gelation, for the former is of ionic reaction in nature, while the latter, being a cohering phenomenon between somewhat stabilized colloid particles, demands not a small activation energy.

IV, 4. Thermal mutation of Eumyosin

As already mentioned, eumyosin existing in contact with water undergoes spontaneous changes in the lapse of time so as to become according to circumstances either difficultly-soluble in neutral salt solutions or readily-soluble in plain water (*cf.* III, 3, 1; III, 3, 4). Moreover, these changes, being distinguished in that they become prompt first after a longer or shorter duration of induction period, point to that there exist in their course some resistances having to be overcome by activating the external and /or internal thermal motions of eumyosin particles before they undergo such changes. At a given temperature, however, the stubbornеss of the resistances seems to be dependent upon the ionic condition of the system. If the particles are provided with a great deal of net charge, they may be prone to suffer thermal disintegrations, whereas they may be prone to undergo thermal coagulation if they are poor in net charge.

IV, 4, 1. Thermal Disintegration

The experimental results treated in III, 2, 6 suggest that eumyosin particles, when imposed with an appreciable amount of net charge, are liable to be submitted to thermal disintegration into far smaller units which are no more capable of restoring the original particles. In these cases, it may be probable that some intramolecular changes are brought about; for instance, the observed shift of the point of minimum solubility and/or minimum viscosity towards a more acid pH to the original isoionic point (*cf.* III, 3, 3) points to the multiplication of α-carboxyl groups, while hydrolytic break-down of cystine bridges into sulfohydryl groups and sulfenic groups might also be effected under certain circumstances.

It is likely that the intramolecular repulsion exerted by excessive net charge renders the molecules to be expanded so extremely that they are readily disintegrated when their thermal vibration grows vigorous. However, since a more or less appreciable amount of intact eumyosin can be recovered from an eumyosin-NaOH sol as long as its intrinsic viscosity does not become less than about 0.2, it is inferred that the intramolecular disintegration can take place exclusively with isolated molecules. Presumably, the molecules buried entirely within a micelle will not directly be subject to disintegration on account of the comparatively large inertia of the micelle as a whole.

In pH ranges of medial acidity or basicity where eumyosin molecules are bound together into micelles not intensively but extensively on account of that a large number of atoms belonging to the interacting molecules are kept in

contact with each other by rather weak attraction forces, the thermal disintegration will also advance at a medial rate, since the micelle-constituting moleculs will be set free into singular molecules rather with ease.

Remarks 5, Thermal denaturation. An isolated free molecule of eumyosin, even if it is not submitted to any drastic thermal disintegration such as hydrolysis or deamination, is liable to be modified in its intramolecular structure on account of its small inertia against thermal vibration. If the helical molecular chain is once extended beyond a certain limit so that its constituent reciprocally-adjecent segments jump apart from each other into another potential trough, it will no longer restore the genuine orderly arrangement of the chain segments, and will be reformed into a far more probable, randomly-convoluted, balled-up chain when the tension is put away. This confusion in intramolecular structure brought about by heat may presumably constitute the thermal denaturation of eumyosin.

When eumyosin undergoes thermal denaturation, its dipolar moment will be diminished on account of that the orderly arrangement of ionic groups is abolished permanently; this will accordingly cause eumyosin to recede in its ability of salting-in. Moreover, since the intramolecular recurrence symmetry of its intact molecules, which crystallizes the latter into fibrilar micelles, is lost too, this will give rise furthermore to their loss of gelating ability.

Remarks 6. Despite of that psudomyosin is in general detectable only in alkali extracts of bleached fish, the thermal disintegration seems not to be a mere implication of further separation of pseudomyosin from eumyosin, because the reaction product never exhibits any high viscosity such as revealed by intact pseudomyosin and/or eumyosin. It seems interesting that the pH of the minimum solubility of the thermal disintegration product, pH 4.6, is situated closely to the reported pH of the isoelectric point of actin, pH 4.7 (Szent-Györgyi, 1947), for eumyosin may be looked upon as identical in nature with actomysin which is regarded, in turn, as a complex of myosin-A with actin.

IV, 4, 2. Thermal Coagulation

As informed from experimental data (*cf.* III, 4), whilst eumyosin freshly precipitated is readily salted-in in neutral salt solutions, it becomes spontaneously more and more difficultly-soluble in such if kept precipitated, especially when concentrated further by straining.

The extent to which an eumyosin deposit becomes difficultly-soluble is considered to depend not only upon the intensity but also upon the magnitude of the area of contact established between eumyosin fibrils constituting the deposit. Since, as generally accepted, the atoms lying on the surface of the fibrils should stand at a higher energy level compared with those lying in the interior of the fibrils, thinner fibriles are expected to be always striving to cohere to form thicker ones. Actually, however, the resultant of the intermolecular attraction forces being considered to be of van der Waals-London nature and the repulsion forces due to the net charges on individual molecules determines the final state of aggregation between the fibrils, into which thermal agitation leads them more or less readily according to temperature. Because these two kinds of forces are both of additive nature, if any attraction is prevailing at all

as their resultant, the attraction will become more and more strong with the growth of fibrils in so far as their dimensions remain within a colloidal region.

On the other hand, it will be easily understood that when thin fibrils being previously oriented at random are aggregated into a bundle, the effective space occupied by the crowd of these fibrils is necessarily reduced in volume. It follows, therefore, that a jelly whose network structure is constituted of thin fibrils contracts as a whole, as such bundling-up of fibrils takes place here and there throughout the bulk of the jelly, squeezing water out of it. This may be the phenomenon of syneresis.[42)]

Thixotropic jelly of eumyosin becomes non-thixotropic and more or less hardly-accessible to the action of peptizers when heated to undergo syneresis. With the advance of syneresis, it is likely that so much number of atoms on a fibril come into contact with such on other fibrils, surmounting the repulsion barrier and jumping into a deep potential valley, that the polar loci on these fibrils are no longer sufficient in number to resolve the fibrilar bundles even when these loci are made to be repulsive centers by the addition of a peptizer.

At the isoionic point, however, there would be no repulsion barrier between the interacting fibrils except the weak hindrance due to the hydration layer; and they might be held together maintly by salt-bridging which is readily dissociable according to the ionic or dielectric condition of the environment. Hence, the isoionic precipitate is peptized rather easily even after it is desiccated (*cf.* III, 3, 4, 4) despite that thermal coagulation of eumyosin is usually fostered by concentrating the protein system.*

Remarks 7. As mentioned in III, 2, 8, eumyosin flocculums compressed by a strong centrifugal force exhibited a second minimum in sediment volume, somewhat indistinct as it was, at pH 8.5. It has already been discussed that the fibril-formation tendency of eumyosin attains to a maximum at this pH. From the abovementioned point of view that syneresis will be a phenomenon characteristic to fibrous colloids, it seems very reasonable to ascribe this second minimum in sediment volume to the prevalence of syneresis in the eumyosin deposit at pH 8.5.

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42) The view that the thicker the fibrils constituting the jelly, the more readily they cohere with one another to form thicker bundles seems to conform to the fact that syneresis proceeds in general along a course like that of an autocatalytic reaction. After the fibrils have become sufficiently thick, the jelly will cease to contract any more, because the bundles of fibrils, being of course oriented at random, do not contract lengthwise; and hence some large quantity of water remains as usual occluded within the mass of jelly even after its syneresis has come to an end. It should be thus expected that syneresis of a jelly is always accompanied by the thickening of its constituent skeletons, *i.* e., the decrease of their surface area but not by uniform narrowing of the spacing between the skeletons.

From this reasoning, it may also be predicted that, when concentrated neutral eumyosin undergoes thermal coagulation in the presence of NaCl, the reaction of the system becomes acid, for the concentration of free NaCl rises due to *Adsorptionsrückgang* (FREUNDLICH, 1930) which proceeds with syneresis so as to shift the reaction towards the isoionic point (*cf.* SÖRENSEN, 1917).

* In making this discussion, the author is indebted to Dr. T. SHIMANOUCHI of *Tokyo* University for his advice.

An apparently very analogous phenomenon that a second minimum is explicitly detectable at a different pH to the isoelectric point has early been reported on the swelling of gelatin gels by Wilson and Kean (1922), and by Rudsit (1930). The interpretations of the phenomenon given by these authors, however, seem by no means to be convincible: Wilson and Kean have considered that between pH 4.7 and 7.7 the gelatin passes from the gel to the sol form and that the second point of minimum occurred at the isoelectric point of the sol form. On the other hand, Rudsit, assuming the effect of some antiswelling action of H^+ ion or OH^- ion, has proposed that the action may become maximal at a slightly acid or basic reaction, respectively, to the neutrality, while he had made no comment to the origin of such antiswelling forces. The present author is of opinion that this apparent second minimum occurrence in the swelling of gelatin may certainly be due to that the gelatin underwent syneresis through quite a similar mechanism to the case of eumyosin jelly.

Remarks 8. With aqueous concentrated systems of eumyosin precipitate which were kept in a refrigerator driven by an electric motor, it was often experienced that colonies of the protein flocculum became redistributed throughout the system so as to develop a notable feature wherein they were locally concentrated into a number of layers with some regular spatial periodicity. The established feature of such layers are sketched diagrammatically in Figure 20. The thickness of these layers was fund almost always to be 10 *mm* or so, while every one of them consisted further of finer layers, 1–2 *mm* in thickness.

It seems likely that high frequency vibrations derived from the motor in driving were responsible for this periodic local rheopexy of eumyosin flocculums.

IV, 5. Interpretaion of *Kamaboko*-preparation Processes

Whereas the technical methods for *Kamaboko*-preparation have been popularized since early days in this country, it is only quite a recent affair that the principles underlying these techniques became so as to be attacked in the light of science (Hirano, 1939 b; Migita and Okada, 1952). It has already been pointed out by the present author that the only *Kamaboko*-forming component of fish flesh is eumyosin. From the intimate relation between eumyosin and pseudomyosin, however, it is inferred that, in the usual preparation of *Kamaboko*, these two protein fractions, behaving as a complex, classical myosin, act hand in hand so as to produce the *Kamaboko*-jelly. Though it is not certain what role is played here by the component pseudomyosin, it is presumed that this protein fraction behaves as a sort of buffer against the extraordinarily large gelating ability of eumyosin, which might certainly make the practical operation rather difficult to be controlled.

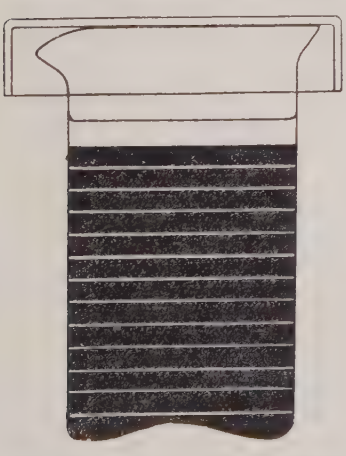

Fig. 20. Poly-layered precipitate of eumyosin.

Now, on what principles stands at all the practical procedure of *Kama-*

boko-preparation? In the following, this question will be given an answer, being based on the behaviors of eumyosin discussed in preceding sections.

As wellknown, the essential stages of *Kamaboko*-preparation process consist of (1) Mechanical crudely-crushing of raw fish, (2) Passing the crushed fish through a colander, (3) Bleaching the strained fish with water, (4) Pressing water off from the bleached fish, (6) Grinding up the pressed fish into a paste under the addition of common salt, (6) Moulding the paste into *Kamaboko*-blocks, (7) Appropriately-setting the paste into an elastic jelly, and (8) Steaming or roasting the jelly blocks. Fundamental significances of these stages will be interpreted as follows:

(1) **Crudely-crushing of raw fish.** By means of this operation, the histological structure of the raw material flesh is destroyed so as to be easily subjected to the following operations, from (2) to (5).

(2) **Passing the crushed fish through a colander.** The aim of this operation is to reject mechanically such tissues as behave objectionably against the paratability of the final product, *e.g.*, bones, scales, and *etc.*, from the fish.

(3) **Bleaching the strained fish with water.** This operation being called "*Nikusarashi*" by *Kamaboko*-makers is effective not only for excluding water soluble or mixible unpleasantly-smelling, -tasting, or -coloring substances from the fish but also for facilitating the preparation of a concentrated precipitate of myosin such as enables us to obtain a substantial product of *Kamaboko*. Since neutral salts contained inherently in the muscular system of the fish are put away more or less completely on bleaching, the hydration of myosin is also diminished. If the bleaching water has previously absorbed CO_2 from the atmosphere, myosin may be precipitated still more completely on account of that such water possesses a pH close to the isoionic point of the protein.

Usual bleached fish is expected to contain beside myosin connective tissue proteins such as collagen and elastin and also small quantities of denatured proteins, for raw materials of excellent freshness are seldom used in *Kamaboko*-manufacturing. These protein fractions, however, seem not to interfere the *Kamaboko*-forming ability of myosin except that they behave as mere diluents against myosin, for it has been pointed out by the author that the rigidity of *Kamaboko*-jelly depends only upon the myosin concentration in the bleached fish of which the jelly is made (HIRANO, 1942 d).

(4) **Pressing water off from the bleaced fish.** By carrying out this process, the myosin concentration in the bleached fish is raised sufficiently so that the fish may be able to be converted into *Kamaboko*-blocks such as not accompanied by an appreciable shrinkage due to syneresis. An excessive pressing, however, often effects the coagulation by concentration (*cf.* III, 3, 4, 2) of myosin, making subsequent manipulations difficult.[43]

43) Strange as it may sound, experienced makers of *Kamaboko* say that, if the excellent products are wanted in summer, they avoid to use raw materials of high freshness, whereas such materials are preferred in winter. Their saying seems to mean that, if the pressing operation is brought about at a high temperature, the coagulation by concentration of myosin is accelerated so excessively that the subsequent mixing of common salt no more effects substantial peptization of the protein; the more fresh the raw materials, the more liable are they to become difficultly-peptizable.

When somewhat degraded fish is used as raw material, water cannot be pressed out with ease, for eumyosin has receded in its precipitability in plain water (*cf.* III. 3, 1, 2). It has been known, however, that even such degraded fish becomes often ready to be set into *Kamaboko*-jelly, though any superior quality cannot be expected for the product, if some dry starch is added to the fish as occasionally practised. It is likely that the dry starch thus mixed absorbs water so as to concentrate the residual myosin in the fish and enables the latter to act so as to concrete whole the system into an elastic mass. (In practice, the mixing of starch is done during process (5) that follows.)

(5) **Grinding up the pressed fish into a paste under the addition of common salt**. In this process any muscular tissue remaining within the pressed fish is finely ground up. Since the common salt added to the fish is mixed throughly with the latter and dissolve in the water contained in it, there takes place the salting-in of myosin, giving rise to a paste with marked stickiness.

The grinding operation should not be continued too long, for the sticky fish paste thus produced tends to undergo ionogelation followed by syneresis which makes the paste to be hardly moulded into sufficiently elastic blocks of *Kamaboko*.

It should also be remembered that the common salt added to the ground fish behaves, of course, as an important seasoning.

(6) **Moulding the paste into *Kamaboko*-blocks**. The sticky paste obtained in the preceding process is now subdivided and piled up on small planks of wood, 12–15 *cm* long and 6–8 *cm* wide in usual, so as to make up unit pieces of manufactures, the *Kamaboko*-blocks.

Since the solidification by syneresis of eumyosin becomes prominent first after an induction period is elapsed, during which the protein system behaves as a sort of fluid or thixotropic jelly, the moulding of paste should be finished during this induction period. The abovementioned comment by experienced *Kamaboko*-makers (*cf.* foot note 43) may be also understood as such that is calling attention to the fact that the induction period is markedly shortened in the hot season of summer, especially with the case of ionogelation of concentrated myosin paste pertaining to raw materials of high freshness.

(7) **Setting the paste into an elastic jelly**. Although this process in usual is not intensionally put into practice by makers who prepare numerous pieces of *Kamaboko* successively in a course of time, it should not be neglected in order to obtain highly-elastic manufactures. During the course of this process, the ionogelation of myosin advances slowly but uniformly throughout the mould of raw *Kamaboko*.

From the point of view of *Kamaboko*-manufacturing technique, the setting of the paste into a jelly with adequate tenacity at a relatively low temperature is profitable in order to avoid unwellcome occurrence of cracks within final *Kamaboko*-blocks on the application of process 8.

(8) **Steaming or roasting the jelly blocks**. The blocks of raw *Kamaboko*-jelly are finally heated by steaming or roasting in order to increase their tenacity and elasticity. This may also contribute to sterilizing the product.

On being thus heated, the *Kamaboko*-jelly sufferes syneresis to a slight

extent. An excessive heating therefore should be avoided, for it often effects an inadequate toughness of the product accompanied by unpleasant bitterness presumably traceable to metamyosin formation.

(to be continued in Part III)

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* for part I (Hirano, 1951).

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POLAROGRAPHIC STUDIES ON THE SMELL OF FISH AND FISH OIL (PRELIMINARY REPORT)

Ikunosuke Okada, Isami Osakabe, Takeaki Kikuchi, Eiji Moriwake

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It can easily be considered that the fish smell component is not caused by a single substance, but several substances are related to it. Nowadays trimethylamine, piperidine and etc. are separated and confirmed to be the component of the fish smell, but most of them are not yet determined in details. At the same time rancid of fish oil is not plain enough, though organic acids, aldehydes, peroxides and etc. are supposed to be the decomposed elements of oil and related with rancid.

In many studies on the determination of fish smell, chromatography is often used, but polarographic method is not yet reported.

Therefore the authors tried the application of polarography on the studies of fish smell and rancid, and have obtained the interesting results. This paper reports the outline of this trial.

When fish meat is distilled with a small quantity of water, the distillate may involve some volatile substances concerned with fish smell. And if the solution shows a polarographic wave, it is considered that the polarographic wave is caused by volatile substances of fish smell.

And as compared with the pure substances which have the same reduction potential in the same supporting electrolytes, the volatile substances are then determined without interference of inorganic matter.

The aeration method is also suitable for this research. In this case the volatile substances are naturally expelled by the air bubbles through the meat solution or oil, and can be caught in a solution without denaturation. And if the solution which involves volatile substances of oil shows a polarographic wave, the volatile substances will be determined in the same manner as the case of the above mentioned distillation method of meat. The authors believe that when the several medium are used at the distillation and aeration of meat solution or oil, and many different supporting electrolytes are used at the polarographic method, the more interesting results will be obtained.

EXPERIMENTAL

The following experiments were tried by polarographic means.

I. Polarographic characteristics of meat distillate.
   For sample;
   (a) Fin whale meat, *Balaenoptera physalus* Linné, (refrigerated).
   (b) Mackerel-pike meat, *Cololabis saira* (Brevoort) were used.

II. Polarographic characteristics of 0.1*N* NaOH solution involved the volatile substances of oil.

For sample;

(a) liver oil of *Deania eglantina* Jordan et Snyder.

(b) liver oil of *Scymnorhinus licha* (Bonnaterre).

(c) body oil of *Balaenoptera physalus* Linné, were used.

Apparatus

The current-voltage curves were recorded by means of the usual polarographic equipment using a mirror galvanometer with full sensitivity of 2.1×10^{-9} amp/mm/m. at 25 ± 0.1°C.

The potential of the polarographic wave was decided by the half wave potential method against saturated calomel electrode.

I. Polarographic characteristics of meat distillate.

(a) 100 g. of Fin whale meat (immediately after melting) was distilled with 30 ml. of water by means of the usual distillating apparatus, then 0.1*N*

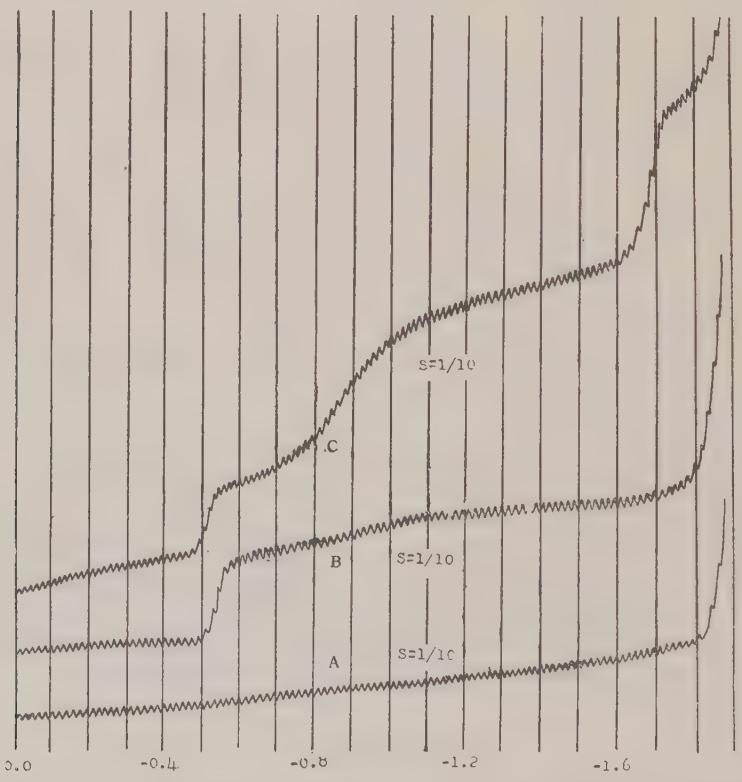

Fig. 1. Polarogram of distilled solution of Fin whale meat *Balaenoptera physalus* Linné. (A) 0.1*N* NaOH solution (control); (B) Distilled solution of Fin whale meat immediately after melting. Supporting electrolyte is 0.1*N* NaOH solution. Reduction potential = −0.7653 volt *vs* S.C.E. Anode potential = −0.1403 volt *vs* S.C.E.; (C) Distilled solution of Fin whale meat elapsed for eight days at room temperature after melting. Supporting electrolyte is 0.1*N* NaOH solution. Reduction potential = −0.7423 and −1.9173 volts *vs* S.C.E. Anode potential = −0.2323 volt *vs* S.C.E.

NaOH solution was added to this distillate of the same quantity. 6 ml. of the above mentioned solution was electrolysed by polarographic means. This solution showed a well defined polarographic wave and its half wave potential was −0.77 volt *vs* S.C.E. as shown in Fig. 1, B.

On the other hand, 0.1*N* NaOH solution (used as control) was tested and as shown in Fig. 1, A, no polarographic wave was obtained.

Therefore, the polarographic wave having a half wave potential of −0.77 volt in 0.1*N* NaOH solution is caused by a volatile substance of Fin whale meat.

When this distillate was added to the McIlvaine buffer solution of PH=7.0 with the same quantity, the polarogram showed also a well defined polarographic wave having a half wave potential of −0.61 volt *vs* S.C.E. as shown in Fig. 2.

(b) The same experiment was tried to Fin whale meat elapsed for eight days at room temperature after melting. This meat had a kind of slightly bad smell. The preparation of this meat was the same as the above mentioned experiment (a), and 0.1*N* NaOH solution was added to supporting electrolyte.

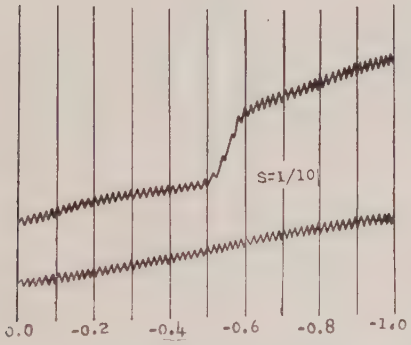

Fig. 2. Polarogram of distilled solution of Fin whale meat *Balaenoptera physalus* LINNÉ. (A) McIlvaine buffer solution of PH=7.0 (control); (B) Distilled solution of Fin whale meat immediately after melting. Supporting electrolyte is McIlvaine buffer solution of PH=7.0. Reduction potential = −0.6093 volt *vs* S.C.E. Anode potential = +0.0493 volt *vs*. S.C.E.

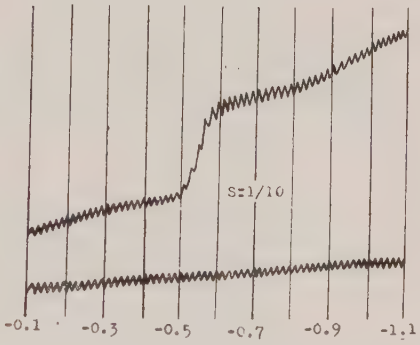

Fig. 3. Polarogram of distilled solution of mackerel-pike meat, *Cololabis saira* (BREBOORT). (A) 0.1*N* NaOH solution (control); (B) Distilled solution of mackerel-pike meat. Supporting electrolyte is 0.1*N* NaOH solution. Reduction potential = −0.8433 volt *vs* S.C.E. Anode potential = −0.3883 volt *vs* S.C.E.

In this case two well defined polarographic waves were produced. One was at −0.74 volt *vs* S.C.E. and another was −1.92 volts *vs* S.C.E. as shown in Fig. 1, C.

The former had almost identical potentical with the case of the above mentioned experiment of (a). Hence the latter could not be found during the experiment of (a), the polarographic wave having a half wave potential of −1.92 volts is more or less regarded as the putrefaction in whale meat.

(c) As a sample, mackerel-pike meat was used. And after the same experiment the same result was obtained, namely the distillate of mackerel-pike meat with 0.1*N* NaOH solution showed also a well defined polarographic wave.

Its half wave potential was −0.84 volt *vs* S.C.E. These results were presented in Table 1.

Table 1. Polarographic characteristics of meat distilled solution (Sensitivity of galvanometer=1/10). * immediately after melting. ** elapsed for eight days at room temperature after melting.

Kinds of meat	Polarogram	Supporting electrolyte	$\pi_{\frac{1}{2}}$ volt *vs* S.C.E.
Fin whale*	Fig. 1, B	0.1*N* NaOH solution	−0.7653
Fin whale**	Fig. 1, C	〃	−0.7423, −1.9173
Mackerel-pike	Fig. 2	〃	−0.8433
Fin whale	Fig. 3	McIlvaine buffer solution of pH=7.0	−0.6093

II. Polarographic characteristics of 0.1*N* NaOH solution involved the volatile substances of oil.

The aeration apparatus, used in this experiment, aimed at the volatile substances absorbed in 0.1*N* NaOH solution, was shown in Fig. 4.

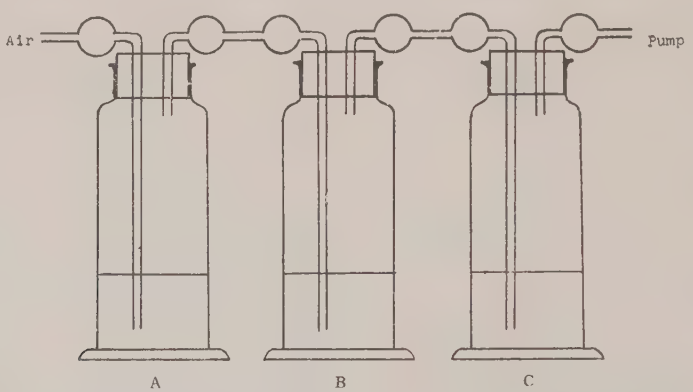

Fig. 4. Aeration apparatus. (A) 10% pyrogaroll in 50% KOH solution; (B) Sample of oil; (C) 0.1*N* NaOH solution.

Air was bubbled through in 100 ml. of 0.1*N* NaOH solution (C), after through 100 ml. of oil (B). Oxygen was removed by 10% pyrogaroll in 50% KOH solution (A).

After 24 hours, 6 ml. of aerationed 0.1*N* NaOH solution was electrolysed by the polarographic means. In this case purified hydrogen gas was passed for 30 minutes. And, as a control, 0.1*N* NaOH solution bubbled through air at the same condition was tested. 0.1*N* NaOH solution involved the volatile substance of *Deania eglantina* J. et S. liver oil by the above mentioned aerationed method showed a well defined polarographic wave with a half wave potential of −1.06 volts *vs* S.C.E. (Fig. 5, C).

And 0.1*N* NaOH solution bubbled through air at the same condition showed no polarographic wave as shown in Fig. 5, A. Therefore the polarographic wave having a half wave potential of −1.06 volts seemed to be caused by a

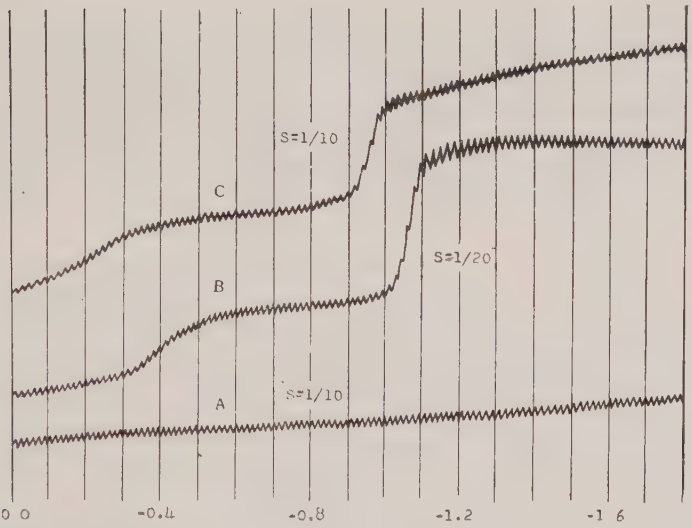

Fig. 5. Polarogram of 0.1*N* NaOH solution involved the volatile substances of *Deania eglantina* J. et S. liver oil. (A) 0.1*N* NaOH solution throughed air (control); (B) 0.1*N* NaOH solution involved the volatile substance of *Deania eglantinia* J. et S. liver oil by aeration method. Reduction potential = −1.0553 volts *vs* S.C.E. Anode potential = −0.0297 volt *vs*. S.C.E.; (C) The lower part of solution when the oil was agitated with 0.1*N* NaOH solution. Reduction potential = −1.0605 volts *vs* S.C.E. Anode potential = +0.1455 volt *vs* S.C.E.

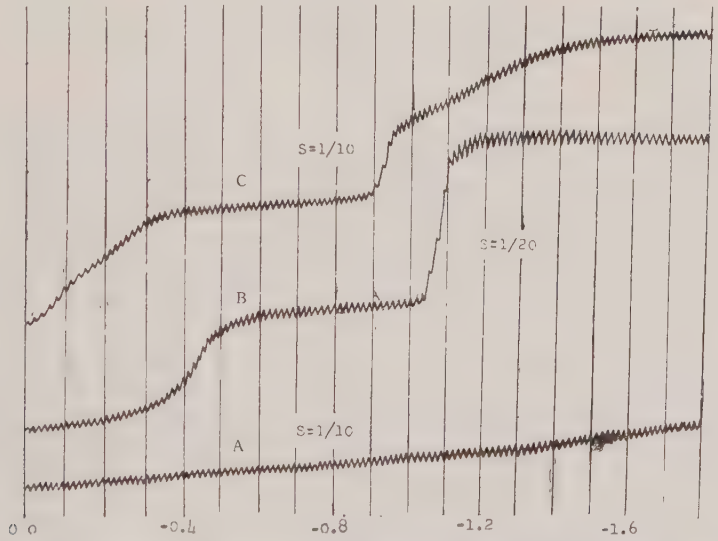

Fig. 6. Polarogram of 0.1*N* NaOH solution involved the volatile substances *Scymnorhinus licha* (BONNATERRE) liver oil. (A) 0.1*N* NaOH solution througed air (control); (B) 0.1*N* NaOH solution involved the volatile substance of *Scymnorhinus licha* (BONNATERRE) liver oil by aeration method. Reduction potential = −1.0533 volts *vs* S.C.E. Anode potential = −0.0367 volt *vs* S.C.E.; (C) The lower part of solution when the liver oil was agitated with 0.1*N* NaOH solution. Reduction potential = −1.0908 volts *vs* S.C.E. Anode potential = −0.0367 volt *vs* S.C.E.

volatile substance of *Deania eglantina* J. et S. liver oil.

Still more, the following experiment was tried. 1 ml. of *Deania eglantina* J. et S. liver oil was agitated with 20 ml. of 0.1*N* NaOH solution about five minutes, and left at room temperature for about one hour, then the lower part of 0.*N* NaOH solution was separated and filtered. This solution also showed a well defined polarographic wave with a half wave potential of −1.06 volts *vs* S.C.E. as shown in Fig. 5, B.

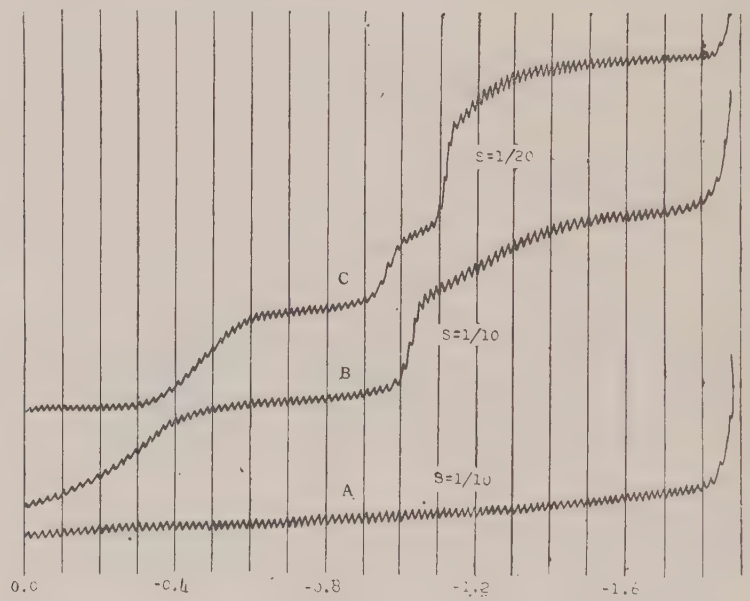

Fig. 7. Polarogram of 0.1*N* NaOH solution involved the volatile substance of *Balaenoptera physalus* LINNÉ body oil. (A) 0.1 *N*NaOH solution (control); (B) 0.1*N* NaOH solution the volatile substance of *Balaenoptera physalus* LINNÉ body oil by aeration method. Reduction potential= −1.1622 volts *vs* S.C.E. Anode potential= +0.1412 volt *vs* S.C.E.; (C) The lower part of solution when the body oil was agitated with 0.1*N* NaOH solution. Reduction potential= −1.1058 and −1.2703 volts *vs* S.C.E. Anode potential= +0.1403 volt *vs* S.C.E.

Table 2. Polarographic characteristics of 0.1*N* NaOH solution involved volatile substances of oil. * 1 ml. of sample oil was agitated with 20 ml. of 0.1*N* NaOH solution about five minutes, and left at room temperature for about one hour, then the lower part of solution was separated and filtered.

Kinds of oil	Polarogram	Preparation	$\pi_{\frac{1}{2}}$ *vs* S.C.E.	sens. of galv.
Deania eglantina J. et S. (liver oil)	Fig. 5, B Fig. 5, C	aeration method 0.1*N* NaOH*	−1.0553 −1.0605	1/20 1/10
Scymnorhinus licha (BONNATERRE) (liver oil)	Fig. 6, B Fig. 6, C	aeration method 0.1*N* NaOH*	−1.0533 −1.0908	1/20 1/10
Balaenoptera physalus LINNÉ (body oil)	Fig. 7, B Fig. 7, C	aeration method 0.1*N* NaOH*	−1.1622 −1.1058 −1.2703	1/10 1/20

This polarographic wave is almost alike to the case of the aeration method (Fig. 5, C). The same experiments were tried with *Scymnorhinus licha* (Bonnaterre) liver oil and *Balaenoptera physalus* Linné body oil, and the same results were obtained.

These results were presented in Table 2.

Thus the polarographic waves which were caused by the volatile substances concerned with fish smell and rancid oil were obtained. And these polarographic waves are considered as the reducible substance concerned with fish smell or rancid oil.

The authors are now trying the several distillation of fish meat in many different media such as acidity, alkalinity and neutral, and at the same time, trying the use of several supporting electrolytes such as KCl, HCl, tetramethyl ammonium salts, many of other buffer solutions and etc. to the distillate of fish meat.

On the other hand in order to determine the component of fish smell and rancid oil, the authors are also trying the investigation of polarographic characteristics of pure substance which are regarded as the component of the fish smell and rancid oil.

And when the pure substances which have the same reduction potential as experimental results in the same supporting electrolyte are found, then the substance concerned with fish smell or rancid oil will be determined.

These results will be reported in latter papers.

Résumé

The polarographic method was applied to the studies of fish smell, and the following investigations were carried out.

(a) Polarographic characteristics of meat distillate.

For sample; Fin whale meat *Balaenoptera physalus* Linné (refrigerated) and mackerel-pike meat *Cololabis saira* (Brevoort) were used. These meat distillate showed a well defined polarographic wave having the half wave potential in the range of −0.74 to −0.84 volt *vs* S.C.E. in 0.1*N* NaOH solution and −0.60 volt in McIlvaine buffer solution of PH=7.0.

These polarographic waves seemed to be caused by the volatile substances concerned with fish smell.

The distillate of Fin whale meat which had slightly bad smell showed another polarographic wave having a half wave potential of −1.92 volts *vs* S.C.E. It seems that the wave is caused by the volatile substance concerned with the putrefaction of Fin whale meat.

(b) Polarographic characteristics of 0.1*N* NaOH solution involved the volatile substance of oil.

For sample; liver oil of *Deania eglantina* J. et S. and *Scymnorhinus licha* (Bonnaterre), and body oil of *Balaenoptera physalus* Linné were used. 0.1*N* NaOH solution involved the volatile substance of oil by aeration method, showed a well defined polarographic wave having a half wave potential in the range

of −1.05 to −1.16 volts *vs* S.C.E. It seems that the waves are caused by the volatile substance of oil or rancid oil.

Finally, the authors wish to express their hearty thanks to Prof. I. Tachi of Kyoto Univ., who helped them in this experiment, and wish also to record their thanks for the financial aid given by the Taiyo Gyogyo Co., LTD. and Nippon-Suisan Co., LTD. for pursuing the present study.

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ON THE STUDIES OF GLUTATHION IN THE MARINE PRODUCTS: PART 2. POLAROGRAPHIC DETERMINATION OF GLUTATHION IN CARP AND WHALE

Ikunosuke Okada, Isami Osakabe, Yuji Kuratomi, Eiji Sekine

(Received Jan. 20, 1953)

Glutathion contained in seaweeds was satisfactorily determined by polarography as reported on the part 1. Then the authors had tried the determination of glutathion in the visceras and meats of the carp and whale.

EXPERIMENTAL

Determination of glutathion by polarography

The current-voltage curves were recorded by means of the usual polarographic equipment using a mirror galvanometer with full sensitivity of 2.1×10^{-9} amp/mm/m at 25 ± 1°C., and a normal mercuro-sulphate electrode was used as an anode, at room temperature. In this case, the potential of oxidation wave was decided by the half wave potential method against the saturated calomel electrode. Standard polarogram of glutathion was shown in Fig. 1, which was obtained in the same manner as reported part 1.[1]

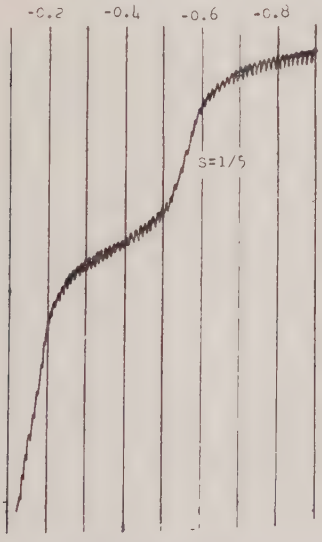

Fig. 1. Standard Polarogram of 2×10^{-4} M Glutathion in McIlvaine buffer solution of pH=7.0 using a normal mercuro-sulfate electrode. Oxidation potential -0.1425 volt *vs* S.C.E. Anode potential $+0.3523$ volt *vs* S.C.E.

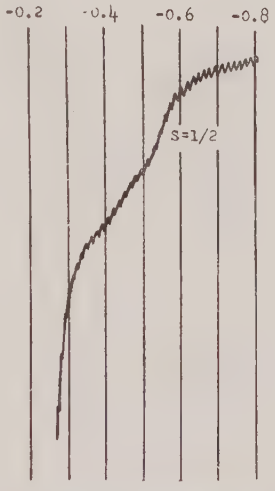

Fig. 2. Polarogram of "Meat" in McIlvaine buffer solution of pH=7.0 using a normal mercuro-sulfate electrode. Oxidation potential -0.143 volt *vs* S.C.E. Anode potential $+0.406$ volt *vs* S.C.E.

Determination of glutathion in carp

The carp used in this experiment was get from a breeding pond of Tama-river side in May 5. 1951, and determined the glutathion, in the meat, bloody fresh, liver, ovary, and spermary.

10 g. of the muscle of carp was crushed with a small quantity of quartz sand, added with 15 cc. of 5% meta-phosphoric acid solution and 15 cc. of Mc-

Table I. The contents of glutathion in the meat and internal organ of carp by polarography. Note: *: McIlvaine buffer solution of pH=7.0 was used.

Sample	Polaro-gram	Solution	Oxidation potential	Height of wave	Sensi-tivity of galvano-meter	Contents of gluta-thion	Mois-ture of sample
Standard glutathion	Fig. 1	2×10^{-4} M *pH=7.0	volt −0.1425	mm 37.7	1/5	mg% 6.14	%
Meat	Fig. 2	Sample 10 g $5\%HPO_3$ 15 cc *pH=7.0 15 cc	−0.143	18.0	1/2	4.42	79.0
Bloody fresh	Fig. 3	Sample 10 g $5\%HPO_3$ 15 cc *pH=7.0 15 cc	−0.135	12.5	1/10	15.35	78.0
Liver	Fig. 4	Sample 10 g $5\%HPO_3$ 15 cc *pH=7.0 15 cc	−0.140	13.5	1/5	8.15	58.0
Ovary	Fig. 5	Sample 5 g $5\%HPO_3$ 10 cc *pH=7.0 10 cc	−0.148	46.0	1/10	34.99	70.0
Spermary	Fig. 6	Sample 10 g $5\%HPO_3$ 15 cc *pH=7.0 15 cc	−0.135	43.5	1/5	25.06	73.0

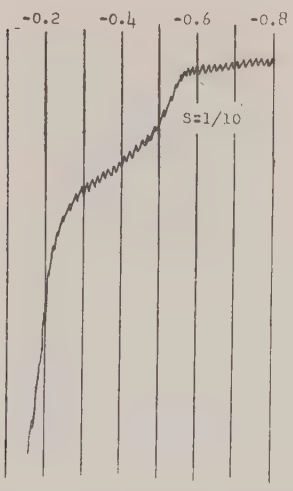

Fig. 3. Polarogram of "Bloody-fresh" in McIlvaine buffer solution of pH=7.0 using a normal mercuro-sulfate electrode. Oxidation potential −0.135 volt *vs* S.C.E. Anode potential +0.385 volt *vs* S.C.E.

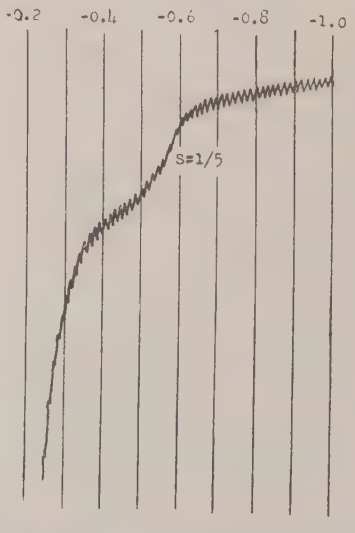

Fig. 4. Polarogram of "Liver" in McIlvaine buffer solution of pH=7.0 using a normal mercuro-sulfate electrode. Oxidation potential −0.140 volt *vs* S.C.E. Anode potential +0.430 volt *vs* S.C.E.

Ilvaine buffer solution of pH=7.0, then stirred, and extracted. The above mentioned extract was electroysed by polarographic method, and the polarogram was obtained as shown in Fig. 2. The height of the oxidation wave of glutathion was 18.0 mm. (Sens. of Galv.=1/2) therefore, 4.42 mg % of glutathion was contained in this meat of carp as compared with the standard glutathion. (The content of moisture in this sample was 79%) The above mentioned polarographic determination was applied to the other parts of carp (bloody fresh, liver, ovary and spermary), and these results were shown in Table 1, and the polarograms (Fig. 3, 6).

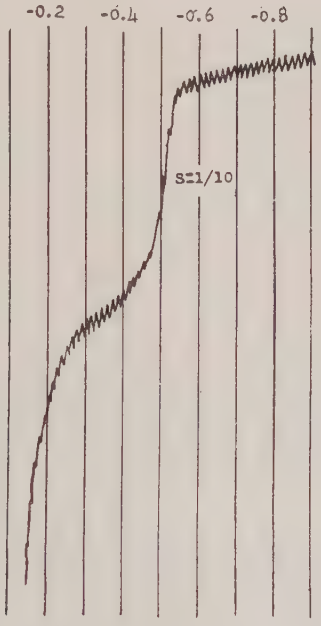

Fig. 5. Polarogram of "Ovary" in McIlvaine buffer solution of pH=7.0 using a normal mercuro-sulfate electrode. Oxidation potential −0.148 volt *vs* S.C.E. Anode potential +0.372 volt *vs* S.C.E.

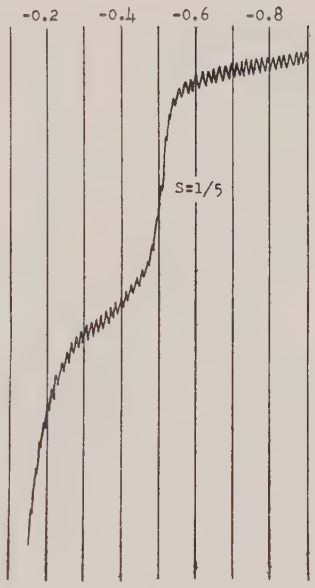

Fig. 6. Polarogram of "Spermary" in McIlvaine buffer solution of pH=7.0 using a normal mercuro-sulfate electrode. Oxidation potential −0.135 volt *vs*vs S.C.E. Anode potential +0.393 volt *vs* S.C.E.

Determination of glutathion in fin whale

The refrigerated samples used in this experiment were offered by Taiyo-Gyogyo Co., LTD. in June 10, 1952, and the glutathion in the meat, liver, and testicle of whale were determined.

10 g. of the meat was crushed with a small quantity of quartz sand, and stirred with 10 cc. of 5% meta-phosphoric acid solution and then filtered, McIlvaine solution of pH=7.0 (equal to the filtrate in quantity) was added to the filtrate. The above mentioned filtrate was electrolysed by polarographic method, and the polarogram was obtained as shown in Fig. 7. The height of the oxidation wave of glutathion was 36.0 mm. (Sens. of Galv.=1/10), therefore, 34.19 mg% of the glutathion was contained in this meat as compared with the standard

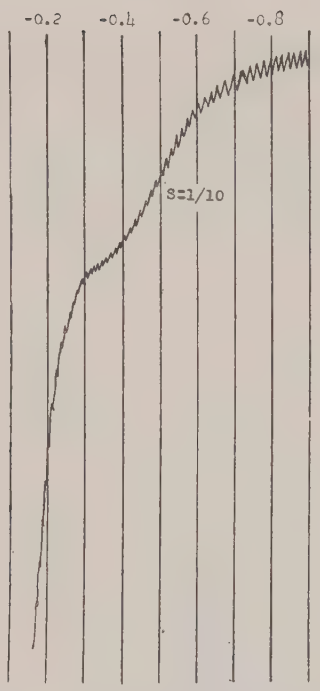

Fig. 7. Polarogram of "Meat" in McIlvaine buffer solution of pH=7.0 using a normal mercuro-sulfate electrode. Oxidation potential −0.115 volt *vs* S.C.E. Anode potential +0.400 volt *vs* S.C.E.

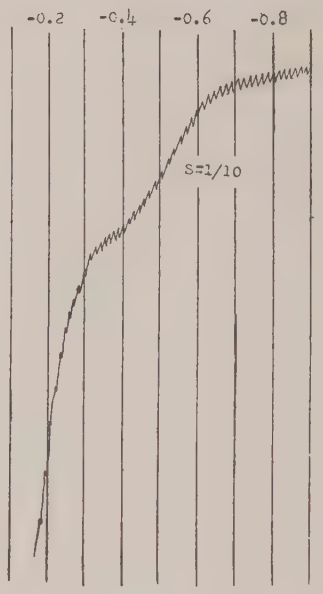

Fig. 8. Polarogram of "Drip of Meat" in McIlvaine buffer solution of pH=7.0 using a normal mercuro-sulfate electrode. Oxidation potential −0.128 volt *vs* S.C.E. Anode potential +0.472 volt *vs* S.C.E.

Table 2. The contents of glutathion in the meat, internal organ, and their drips of whale by polarography. Note: *: McIlvaine buffer solution of pH=7.0 was used, and it volume was equal to the 5% HPO_3 solution extracts.

Sample	Polarogram	Solution	Oxidation potential	Height of wave	Sensitivity of galvanometer	Contents of glutathion	Moisture
Standard glutathion	Fig. 1	2×10^{-4} M *pH=7.0	volt −0.1425	mm 37.7	1/5	mg% 6.14	%
Meat	Fig. 7	Sample 10 g 5% HPO_3 10 cc *pH=7.0	−0.115	36.0	1/10	34.19	73.8
Drip of meat	Fig. 8	Sample 10 g 5% HPO_3 10 cc *pH=7.0	−0.128	27.5	1/10	30.02	—
Liver	Fig. 9	Sample 10 g 5% HPO_3 10 cc *pH=7.0	−0.161	24.5	1/10	22.83	70.7
Drip of liver	Fig. 10	Sample 10 g 5% HPO_3 10 cc *pH=7.0	−0.128	21.5	1/10	23.47	—
Testicle	Fig. 11	Sample 10 g 5% HPO_3 10 cc *pH=7.0	−0.264	11.0	1/5	2.58	85.9
Drip of testicle	Fig. 12	Sample 10 g 5% HPO_3 10 cc *pH=7.0	−0.207	9.0	1/5	3.78	—

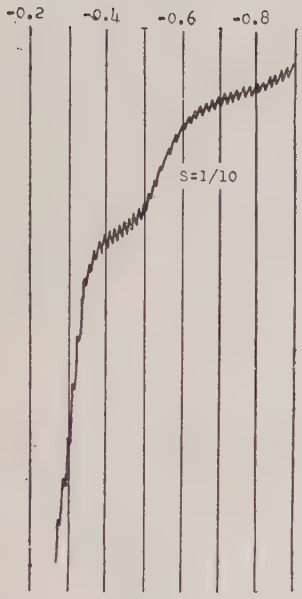

Fig. 9

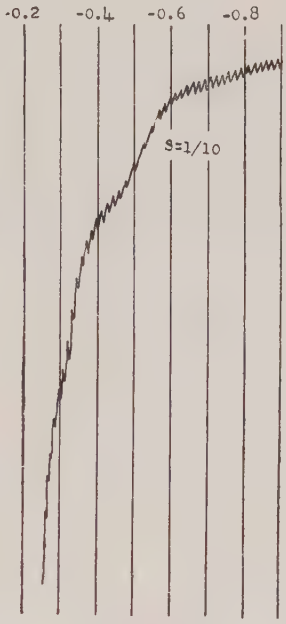

Fig. 10

Fig. 9

Polarogram of "Liver" in McIlvaine buffer solution of pH=7.0 using a normal mercuro-sulfate electrode. Oxidation potential −0.161 volt *vs* S.C.E. Anode potential +0.384 volt *vs* S.C.E.

Fig. 10

Polarogram of "Drip of Liver" in McIlvaine buffer solution of pH= 7.0 using a normal mercuro-sulfate electrode. Oxidation potential −0.128 volt *vs* S.C.E. Anode potential +0.387 volt *vs* S.C.E.

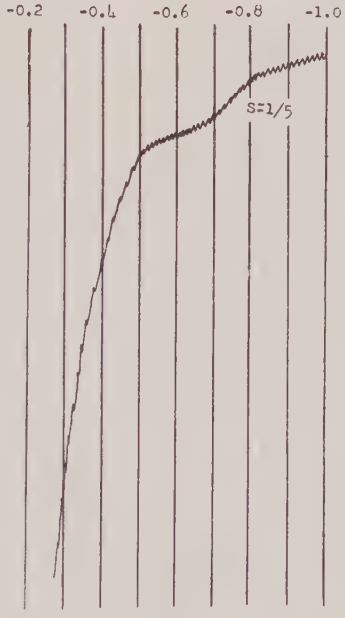

Fig. 11

Fig. 12

Fig. 11

Polarogram of "Testicle" in McIlvaine buffer solution of pH=7.0 using a normal mercuro-sulfate electrode. Oxidation potential −0.264 volt *vs* S.C.E. Anode potential +0.480 volt *vs* S.C.E.

Fig. 12

Polarogram of "Drip of Testicle" in McIlvaine buffer solution of pH= 7.0 using a normal mercuro-sulfate electrode. Oxidation potential −0.207 volt *vs* S.C.E. Anode potential +0.471 volt *vs* S.C.E

glutathion. (The content of moisture in this sample was 73.8%) The above mentioned polarographic determination of glutathion was applied to the liver and testicle of whale, and the results were shown in the table II and the polarograms (Fig. 8~12). According to the results as shown in Fig. 11, 12 (testicle and its drip), these oxidation potentials were differed from that of standard glutathion. But in this case, when a small quantity of the solution of glutathion was added to these samples, the height of oxidation waves were increased at the same potentials as shown in Fig. 13, 14, therefore, it was considered that these waves as shown in Fig. 11, 12, were the waves of glutathion in the testicle and its drip.

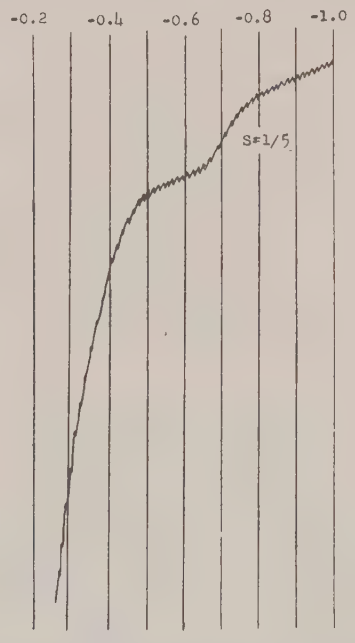

Fig. 13. Polarogram of "The solution of glutathion was added to the testicle" in McIlvaine buffer solution of pH=7.0 using a normal mercuro-sulfate electrode. Oxidation potential −0.267 volt *vs* S.C.E. Anode potential −0.440 volt *vs* S.C.E.

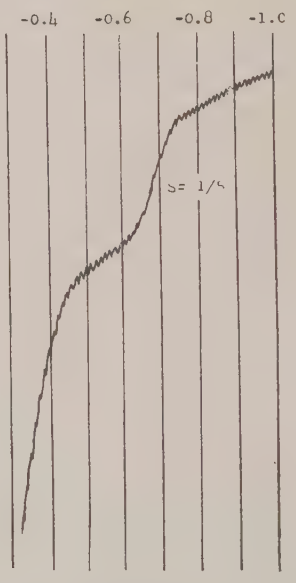

Fig. 14. Polarogram of "The solution of glutathion was added to the drip of testicle" in McIlvaine buffer solution of pH=7.0 using a normal mercuro-sulfate electrode. Oxidation potential −0.204 volt *vs* S.C.E. Anode potential +0.471 volt *vs* S.C.E.

Résumé

1) The polarograms in this experiment indicated the oxidation wave of the reduced type of glutathion.
2) In this experiment, the contents of glutathion in the genital organ were more than that of the other parts of the carp, and the contents of glutathion in the testicle and its drip of whale were very small quantity. It may be assumed that the glutathion contained in the testicle of whale was deteriorated by the refrigeration.
3) As glutathion was contained more or less in the meats and internal organs

of the carp and whale, it may be assumed that it takes charge of the molecular respiration in the living tissue.

4) It has a deep significance that glutathion is contained in the carp and whale, because the sulfohydryl-disulfide compound is important for biological function.

Finally, we wish to express our hearty thanks to Prof. TACHI of the Kyoto Univ., who helped us in this experiment, and also we cannot forget to thank Mr. UMEDA and KUROIWA, ("Kirin" Scientific Research Institute) for their kind offer of pure glutathion. We wish also to record our thanks for the financial aid given by the Ministry of Education, Taiyo-Gyogyo Co., LTD, and Nippon-Suisan Co., LTD, for pursuing the present study.

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ON SIMPLE HYGIENIC EXAMINATION METHOD OF BACTERIA FOR FISH

Yutaka Sekine, Toshio Nakakubo

(Received Nov. 20, 1952)

Introduction

In order to examine fresh fish and other marine products, (Japanese fish pudding) weight method was adopted in 1950.[1] But this method was not proper just to detect bacteria of the surface of fish. In 1951,[2] it was replaced by scratch method, which required a special kind of appliance. But this method also proved not too good.

In the first place, we compared swab method[3] to scratch method. The former is better than the latter as far as detecting bacteria is concerned, but the former is quite similar to the latter on cultivating stage.

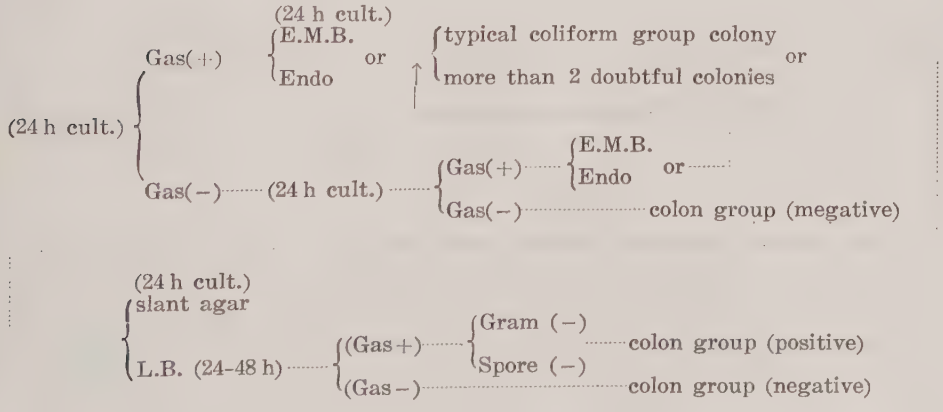
Scratch method takes 5 or 6 days to be gone through while fresh fish should be examined in a very short time. According to the new method we devised in an attempt to shorten the time required, a piece of paper should be pasted right on the surface of fish, and it should be transplanted on the Endo or E.M.B. agar plate. We made a comparison between the new method and the scratch method.

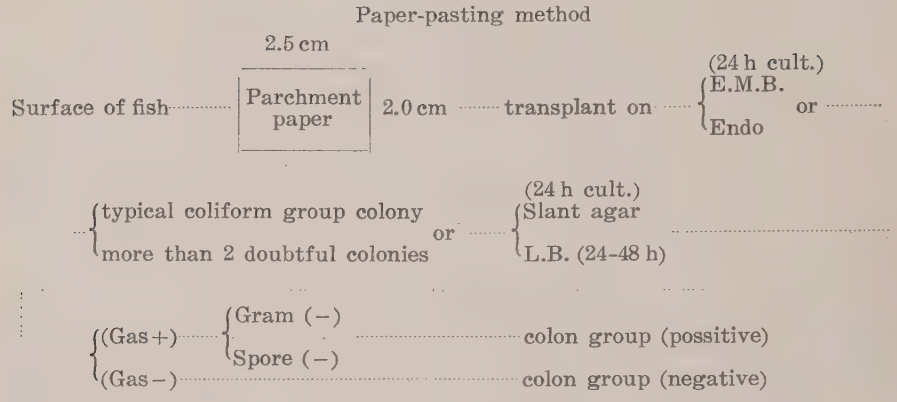
As we think the result is noteworthy, we would like to give a summary here.

The following is the orders of the old method and the new one.

Table 1. Scratch method.

Surface of fish (120 cm²)—NaCl (100 cc)—B. G. L. B. tube (culture)





I. Experimental procedure

1. Paper-pasting method.

First, parchment paper of 2.0 by 2.5 cm in size should be sterilized. If fish is small, medium, or large, 1, 2, or 4 pieces of paper respectively should be pasted on each side of fish, and transplanted on the Endo or E. M. B. agar plate. Since it is not possible to get individual colonies from such phlegmatic fish as cuttle fish, they should be dried in incubator to a certain degree, and after that, the Petri dish should be turned over and put in an incubator. (Size of paper can be various, but the size mentioned above is good when four pieces are pasted on the Petri dish.) (See photograph)

2. Number of fish used for the examination.

As indicated in the Guide,[2] we used 20 small fish, 10 medium-size fish and 5 large fish under the scratch method. Surface of both sides of fish was scratched up.

$2\times3\ cm^2\times20=120\ cm^2$ . . . . . small fish
$4\times3\ cm^2\times10=120\ cm^2$ . . . . . medium-size fish
$8\times3\ cm^2\times\ 5=120\ cm^2$ . . . . . large fish

The area of scratch appliance is square 3 square cm and 120 square cm in total is scratched up. We made comparison between this scratch method and our new method.

small fish 12
medium-size fish 6
large fish 3

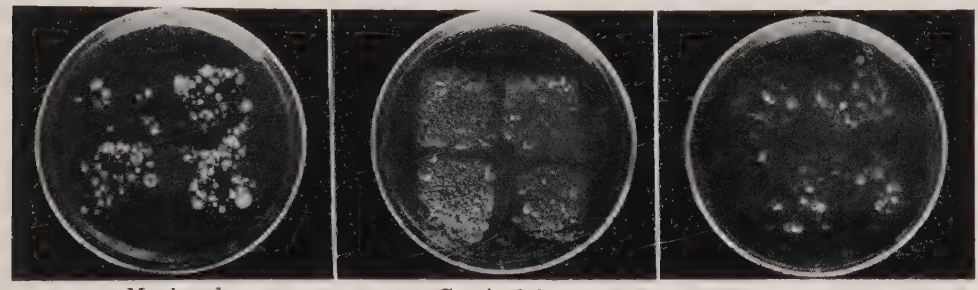
A piece of 5 square cm paper is pasted on both sides

$2\times5\ cm^2\times12=120\ cm^2$
$4\times5\ cm^2\times\ 6=120\ cm^2$
$8\times5\ cm^2\times\ 3=120\ cm^2$

3. Efficiency of the old and new methods.

We tried three examinations upon mackerel by both the old and new methods in order to compare the efficiency of them. We took the average of the three examinations.

First, we washed the surface of fish with running water, then for 5 minutes we dipped it into the 2 litres of water containing 6 milligrams of E. coli cultivated for 20 hours at 37°C on neutral slant agar. After we took it out of the liquid



Mackerel Cattle fish Sardine

we pasted a piece of paper on one side of fish and scrached up the surface of the other side, and we made the both sides of fish 120 square cm.

We put it in the 20 cc of NaCl (0.85%), and shook well. Next, we cultivated 0.1 cc on each of five plates and counted the number of bacteria accurately. We took the average of them to figure out the real efficiency of the two methods.

On the other hand, we cut off the skin of fish which we scratched up and pasted paper on, and put it in the 20 cc solution of NaCl (0.85%). And we cultivated 0.1 cc and counted the number of bacteria. We figured out the total number of bacteria on the surface of fish from which we discovered the efficiency of the two methods.

Table 2. Record of Old and New Methods (by Imvic system).

Kind of fish	Paper-pasting method	Scratch method
Mackerel	Intermediate II type	Intermediate II type
〃	〃 〃	E. coli I type
〃	A. aerog. II type	A. aerog. I type
〃	Intermediate II type	〃 〃
〃	〃 〃	〃 II type
〃	〃 I type	Intermediate I type
〃	〃 II type	〃 〃
Cuttle fish	E. coli I type	〃 II type
〃	〃 〃	E. coli I type
〃	Intermediate I type	Intermediate I type
〃	A. aerog. I type	〃 II type
〃	E. coli I type	〃 I type
〃	A. aersg. I type	E. coli I type
〃	〃 〃	Intermediate I type
Sardine	Intermediate I type	〃 II type
〃	A. aerog. I type	A. aerog. I type
Fish minced and steamed	E. coli II type	Intermediate II type
Fish pudding	None	None

II. Experimental results

1) Comparison of the old method and new our method in efficiency for detecting bacteria.

As a result of a comparison made between the new method and the old one which was conducted by using B. G. L. B. tube, there was found little difference between the two as to the efficiency for detecting colon groups. We also found little difference between the two as to the kinds of coli type found.

2) As shown in the Table No. 3, the result of the comparison of the efficiency made it clear that scratch method is better than paper-pasting method in detecting bacteria, the percentage of which is 20% for the former and 14% for the latter. There was little difference between the two methods in efficiency.

Table 3. Efficiency of Old and New Methods (average of 3 tests).

Mackerel	Paper-pasting 18	Remainding bacteria 114	Efficiency 14%
〃	Scratching 27	Remainding bacteria 117	Efficiency 20%

Conclusion

1) The paper-pasting method is simple in operation and does not need B. G. L. B. fermentation tube, but the Endo or E. M. B. agar plates are used instead.

2) Take with you only sterilized paper and Petri dish to the field, and after cultivated bacteria on Petri dish, bring it back to the laboratory and put it in an incubator immediately,

3) The time necessary for culture is 3 or 4 days, 2 or 3 days less than the old method took.

4) 4 pieces of paper of 2.0 by 2.5 cm in size can be used for culture on a Petri dish, which, we think, is the right size.

5) Instead of M. P. N. computation, we adopted the following method:

When one one coli group colony has been found on a piece of paper, index be 1, and if there were two, then index be 2. In other words, we counted the number of colonies found.

6) We found that the new and old methods were the same in efficiency. Therefore, we think the new method which is simple in operation and takes less time is better than the old.

7) In order to make examination by the new method, we used for sampling 20 small fish, 10 medium size fish or 5 large fish.

Total space is 200 cm^2.

$2 \times 5\ cm^2 \times 20 = 200\ cm^2$ . . . . . small fish
$4 \times 5\ cm^2 \times 10 = 200\ cm^2$ . . . . . medium size fish
$8 \times 5\ cm^2 \times 5 = 200\ cm^2$ . . . . . large fish

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2. Hygienic Examinations Guide 1951.

3. Research on Method of Hygienic Examinations. Research subjects of welfare science, Welfare Ministry. Detection method on contaminative bacteria of fishes. April 1951, pp. 9-12.

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AGE-DETERMINATION OF THE *BABYLONIA JAPONICA* (REEVE), AN EDIBLE MARINE GASTROPOD, BASING ON THE OPERCULUM

Itsuo KUBO and Kei-ichi KONDÔ

(Received Dec. 20, 1952)

Babylonia japonica (REEVE), an ivory shell, is a marine gastropod commonly found in Japan. The meat is useful for our food, and the shell is utilized as the material of toys. Also this animal contributes of late as a material for canning. Neverthless no study has been done of this animal from stand point of the fishery-biology, but INO's one.[1,2] INO has studied on the life history and the method of propagation of this animal. We have carried out a scrutiny for age-determination of the animal dealing with its operculum as the operculum is furnished on outer surface with many growth lines pre-eccentric in distribution.

Before proceeding further we wish to express our hearty thanks to Dr. T. INO, Messrs K. TAKAGI, K. TSURUOKA, and J. TANAKA for their kindness extended during the course of the present investigation. Dr. INO kindly afforded a fine photograph of the fishing gear of this animal. Acknowledgment is also made to the Ministry of Education from which the present investigation has undergone the grant in aid for foundamental scientific research.

(1) Material and the Method for collection

The material, on which the present investigation is chiefly based, was taken on May 16, 1952 from the shallow waters in front of the fish market of Kominato, Chiba Prefecture by a fishing gear. The material consists of 229 individuals ranging 14.0–69.0 mm in shell-length. In addition to this, two sets of materials were also used. They are made up of 669 and 602 individuals measuring 10.0–71.9 mm and 10.0–77.5 mm in shell-length, obtained from the same locality given above on October 5-6, 1951 and July 9–12, 1952 respectively. The shell-length compositions of these material are given in the Table 1.

The fishing gear by which the material were fished is a bamboo basket,

Table 1. Frequency distributions of shell-length.

Date on which the material obtained	Shell-length in mm														
	10 | 12	12 | 14	14 | 16	16 | 18	18 | 20	20 | 22	22 | 24	24 | 26	26 | 28	28 | 30	30 | 32	32 | 34	34 | 36	36 | 38	38 | 40
May 16, 1952			1		1		2	7	3	10	13	16	10	5	3
July 9, 16, 1952	4	29	44	23	4	3	1	5	3	6	1	3	1	4	4
October 5-6, 1951	18	1	1	4	10	17	6	16	21	15	9	8	7	6	6
Total	22	30	46	27	15	20	9	28	27	31	23	27	18	15	13

40–42	42–44	44–46	46–48	48–50	50–52	52–54	54–56	56–58	58–60	60–62	62–64	64–66	66–68	68–70	70–72	72–74	74–76	76–78	Total
13	7	4	5	10	11	9	17	22	12	18	11	10	7	2					229
4	4	7	6	14	39	51	53	68	71	51	35	35	18	7	2	1		1	602
7	10	12	14	18	20	27	38	60	80	77	57	55	28	16	5				669
24	21	23	25	42	70	87	103	150	163	146	103	100	53	25	7	1		1	1500

Fig. 1. Illustration of fishing gear. a, bamboo ring; b, bamboo skewer, to which the baits are fixed; c, buoy. Photograph by Dr. T. Ino.

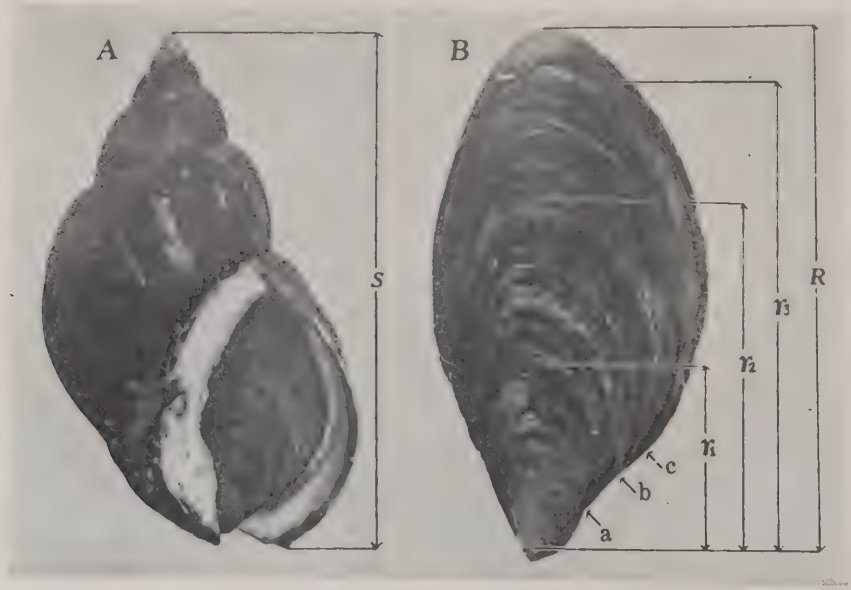


Fig. 2. Oral view of *Babylonia japonica*, 59 mm long (A), and schematic showing of measuring method of the operculum, 28 mm long (B). a–c, growth steps; S. shell-length.

15 cm in mouth-diameter, 20 cm in bottom-diameter, and 17 cm in height,[1] having a stone or other of moderate size within it for weight as shown in the Fig. 1. A bamboo ring is fixed along inner side of the mouth of the gear keeping a distance of about 2 cm from the edge of the mouth (Fig. 1, a). By this ring the shells once entered into the gear are prevented to go out again at least in the shells larger than 2 cm in thickness.

At fishing the gear lowered into the sea bottom with bait, two or three pieces of raw fish, fixed on a bamboo skewer (Fig. 1, b). Then the shells of all sizes come out from the sand of the sea bottom as they are induced by the bait, and climb up along the outer surface of the bamboo basket. And then they enter into the gear going down over the edge of the mouth of the gear. Thus it may be said that there is almost no selection caused by the fishing gear.

(2) Shape of the operculum

The operculum of this shell is obovate leaf-shaped. This belongs to the prenuclear type of the lamellate type according to the classification made by TAKI.[3] The operculum carries a nucleus near its anterior end of the outer surface and a succession of many growth lines laid down eccentric to the nucleus. Growth steps are seen on the outer antero-lateral margin as shown in Fig. 2, a, b, and c. In addition to these, it is provided with growth and resting zones. The resting zones are bearing ill defined or hardly defined growth lines, and are found depressed in comparision to the growth zone. Finally it should be added herewith that the feature of the apparatus in question is undergone no modification by sex.

(3) Measuring method

Measurement has made on the following three items: s, R, r_1-r_n (Fig. 2, B). S represents the length of the shell measured from top of the shell to distal end of the mouth. R that means opercular length, is the distance measured from the nucleus to the posterior end of the operculum. The tertiary item, r_1-r_n, is showing the distance between the nucleus of the operculum and posterior rim of each growth zone of it.

(4) Reading method of the resting zone

At reading the resting zones found on the outer surface of the operculum, two cases were set. In a case of them, all recognizable resting zones were read out without selection and they are writen as r_1, r_2, ...r_n. In the other case, strongly impressed resting zones only were read out consulting with the growth steps lying on the outer antero-lateral edge of the operculum, and they are described under the symbols of r_1', r_2', ...r_n'.

The objectivity of the reading is represented by percentage of coincidence of the resting zones read out by two persons on 20 opercula. At first five persons read out the zones of all the opercula respectively. Subsequently the percentage is obtained comparing two series of the data, each of which is that

Table 2. Coincidence percentage of resting zones read out by two persons on 20 opercula. a, frequency of coincidence; b, total frequency; c, per cent. A, B, ..., E, name of persons engaged for reading of the resting zones; A, K. KONDO; B, K. TSURUOKA; C, J. TANAKA; D, I. KUBO; E, K. TAKAGI.

Name of reader	All resting zones read aut (r)			Strongly defined resting zones only (r')		
	a	b	c	a	b	c
A B	105	145	72.4	27	33	81.8
A C	120	173	69.4	28	33	84.8
A D	80	124	64.5	25	34	73.5
A E	78	122	63.9	24	31	77.4
B C	128	181	69.1	29	33	87.9
B D	87	131	66.4	25	33	75.8
B E	84	129	65.1	25	33	75.8
C D	92	180	51.1	27	33	81.8
C E	85	174	48.9	25	31	80.6
D E	83	96	86.5	26	30	86.7
Total	939	1455		261	324	
Average			64.5			80.6

taken by each different person. As shown in the Table 2, the mean of the objectivity is 64.5% and 80.6% in r and r' respectively.

(5) Relation between lengths of operculum and shell

The relationship between operculum-length (R) and shell-length (S) is linear as shown in the Fig. 3. The regression line may be represented by the following formula, in which the constants of the formula were obtained by least square method :—

$$S=1.91R+1.1 \quad \ldots\ldots (1)$$

Thus both R and S may be considered to be simply proportional. So that they are as follows :—

$$\frac{S_n-\alpha}{S-\alpha}=\frac{R_n}{R}$$

$$S-\alpha=\frac{R_n}{R}(S-\alpha)$$

$$S=\alpha+\frac{R_n}{R}(S-\alpha) \quad \ldots\ldots (2)$$

The constant α shows the shell-length at which the operculum firstly issues, and its value is 1.1 mm according to the formula (1) given above. This value appears to be well in agreement with the actual shell-length judging from the following matters known as the result of the life history investigation of this animal carried out by INO.[2] As the result of INO's investigation, it has been known that the egg of the shell is globular in shape measuring about 0.5 mm in diameter, and that the veliger larva of the animal in 20th dags after hatching has been provided with an operculum developed already.

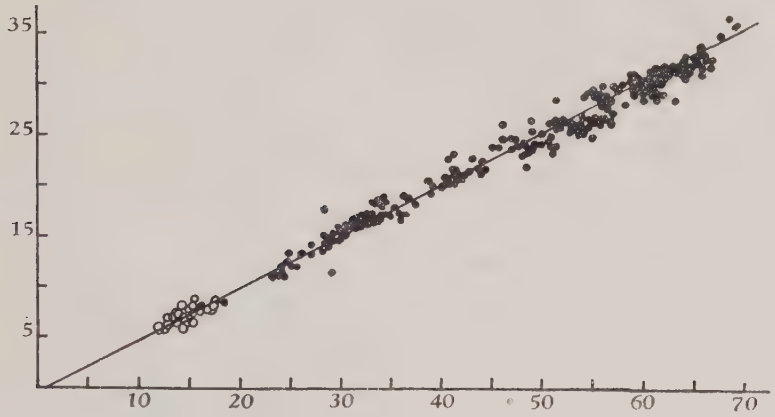


Fig. 3. Relation between lengths in mm of the operculum and the shell (Horizontal axis). Solid circles, based on the material taken on May 16, 1952; soft ones, based on 46 individuals secured on July 11, 1952.

(6) Relationship between number of resting zone and age of the animal

Firstly in regard to r, the frequency distributions of 2 mm-length* group of each resting zone number in relation to the operculum length are given in the Table 3 and Fig. 4. Both average and standard deviation of r_1-r_6 are listed in the Table 4. The range of variation shows a trend successively increasing in order of r_1, r_2, r_3, and r_4, except the cases of r_5 and r_6 in which the sample

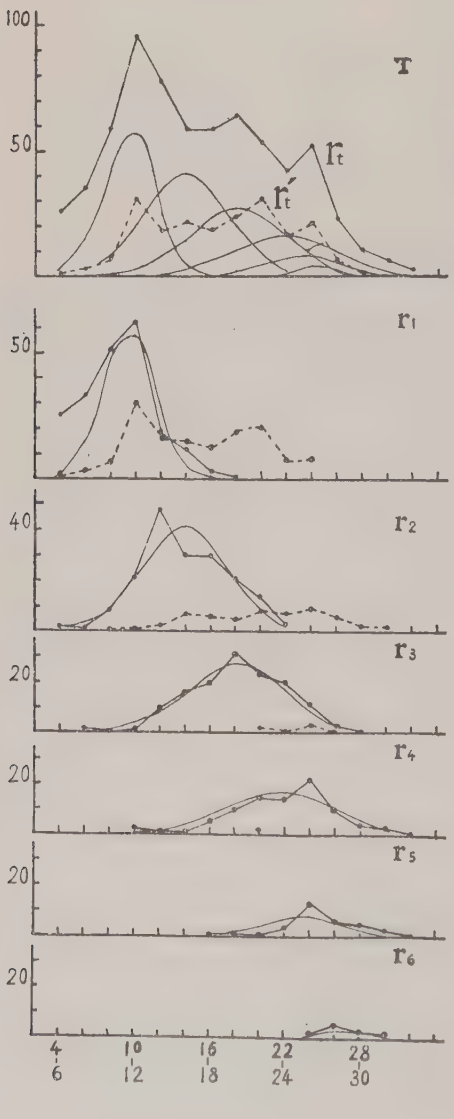
Table 3. Frequency distribution of resting zones in relation to operculum-length. r, all resting zones; r', very legible ones only.

Resting zone	Operculum length in mm															Total
	4 | 6	6 | 8	8 | 10	10 | 12	12 | 14	14 | 16	16 | 18	18 | 20	20 | 22	22 | 24	24 | 26	26 | 28	28 | 30	30 | 32	32 | 35	
r_1	25	33	51	72	19	12	3									216
r_2	1	1	8	21	48	30	30	21	14	3						177
r_3		1	0	1	10	16	20	31	24	20	12	3	1			139
r_4				2	1	1	5	10	15	14	22	10	4	3	1	88
r_5							1	1	1	3	13	6	5	3	1	34
r_6											2	5	3	1		11
r_7													1	0	1	2
Total	26	35	59	96	78	59	59	64	54	40	49	24	14	7	3	667
r_1'	1	3	7	30	17	15	13	19	21	8	9					143
r_2'			1	1	2	7	6	5	8	7	9	6	2	2		56
r_3'									2	1	3	1				7
r_4'											1					1
Total	1	3	8	31	19	22	19	24	31	16	22	7	2	2		207

* Analyses were also made as to those cases: 3 mm- and 4 mm-length groups. But 2 mm-length group seems to be the best for the analysis of this subject.

Table 4. Mean ($\bar{r}$) and standard deviation (SD) of r, mean ($\bar{S}$) and $\bar{S}\pm 2SD$ of the shell-length corresponding to each r. Unit mm.

r	$\bar{r}$	SD of r	$\bar{r}\pm 2\,SD$	$\bar{S}$	$\bar{S}\pm 2\,SD$
r_1	10.37	2.82	4.73~16.01	20.90	10.13~31.67
r_2	14.99	3.46	8.07~21.91	29.73	16.51~42.94
r_3	19.18	3.94	11.30~27.06	37.73	22.68~52.78
r_4	23.00	4.18	14.64~31.36	45.03	29.06~60.99
r_5	24.50	3.30	17.90~31.10	47.89	35.28~60.50
r_6	34.58	2.06	20.46~28.70	48.04	40.17~55.91

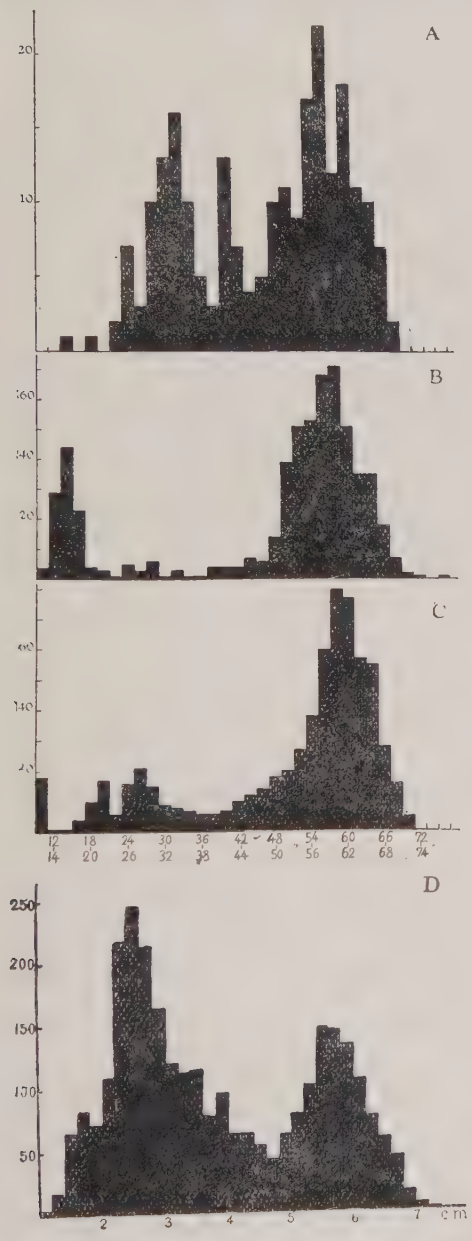


is not sufficient in number. The averages of r_1—r_6 are 10.37 mm, 14.99 mm, 19.18 mm, 23.0 mm, 24.50 mm, and 24.58 mm respectively. And the range of variation ($\pm 2\,SD$) of them runs 4.37–16.01 mm, 8.07–21.91 mm, 11.30–27.06 mm, 14.64–31.36 mm, 17.90–31.10 mm, and 20.46–28.70 mm respectively. These may be converted respectively to shell-length owing to the formula (1) as given in the following lines: 20.90 mm in average (10.13–31.67 mm in range) in r_1, 29.73 mm (16.51–42.97 mm) in r_2, 37.7 mm (22.68–52.78 mm) in r_3, 45.03 mm (29.06–60.99 mm) in r_4, 47.89 mm (35.28–60.50 mm) in r_5, and 48.04 mm (40.17–55.91 mm) in r_6.

In the next place, the frequency distributions of r' in relation to operculum length are given by broken lines in the Fig. 4. The frequency distribution of r_1' is bi-modal having peaks at 10–12 mm and 20–22 mm of the operculum length. The first peak completely agrees with that of r_1. It is irrational that the presence of the second peak. But it seems to the present writers that the presence of the second peak is due to that r_2' in truth has been read out as r_1' because of that the r_1' of it is not well defined owing to the physiolo-

Fig. 4. Frequency distributions of the position at which the resting zones of the operculum appear. Horizontal axis, distance from the focus to the posterior border of the operculum in mm. Broken lines, r'. Smooth curves, normal curves fitted, T, total.

gical conditon of the time in which it is achieved. In r_2', although the mode of the frequency distribution does not be well defeind as the number of sample is meagre in addition to that it is considerably variable with a large rang of variation, the mode appears to lie at about 24–26 mm on the operculum-length. Also the second peak of r_1' is referrable to r_2' by the reason afore mentioned. Then the frequency distribution of r_2' involving the samples of the second peak of r_1' is seen in Fig. 4, r_i'. And its average seems to stand at about 22–24 mm of the operculum length. Hence this coincieds with the mode of the r_4. Accordingly it appears to be that the r_1' and r_2' were achived at the time of 20.90 mm and 45.03 mm in shell-length respectively.



According to the INO's investigation, two independent groups have been found to exist, as seen in the Fig. 5, D and others, on the Petersen-curve of the shell-length based on a sample of about 3,000 individuals gathered from Okitsu, Kominato, and Amatsu of Chiba Prefecture during a period from a time a little before the breeding season to the height of it (from May to July). The mode of these two groups lies at those positions of 25 mm and 56 mm respectively. Of these groups, the first group, as already pointed out by INO, may be supposed to belong to one age group of full year, and the second one deduced to consist of two-age and more-age classes of full year by the reasons given in the following lines, viz., the material on which the Petersen-curve of the shell-length is based, is that caught during spaw-

Fig. 5. Frequency distributions of shell-length (Horizontal axis) in mm of *Babylonia japonica*. A, based on 229 shells secured on May 16, 1952; B, based on 602 individuals taken on July 9–12, 1952; C, based on 669 ones obtained on October 5–7, 1951; D, reproduced from INO's paper, the figure is based on ca. 3,000 individuals taken during a period from May to July of 1946.

ning season, and the growth of the larva of the shell owing to the culture made by INO in the laboratory is 3.25 mm in shell-length in one month, 4.8 mm in two months, and 10. 8 mm in 4 months after hatching. Also this animal is usually fished until November, but it becomes not fished after December as it dives into sand. So that this animal seems to hibernate from such season with cessation of feeding. Because of such ecologycal respects given above the resting zones of r' appear to be made up in the winter season* atfer December. As the summary of the matters mentioned above, it is said presumably that the group of this animal with shell-length of about 21 mm on average is of about half year after hatching, and that with shell-length of about 45 mm on average is of about one and half years after hatching. The differences found between the averages of shell-length when the resting zones of r_1' (or r_1) and r_2' (or r_4) of the present material are formed and those of the first and second groups of INO's material are referable to the differences of the growths of them during a period of half year respectively. In this place the difference found between the latters is considerably larger than that found between the formers. This is probably due to that the INO's second group comprises the shells of 3- or more-ages in addition to those of 2-age.

Lastly it should be added herewith that there is r_2 and r_3 lying between r_1' and r_2' (or r_1 and r_4) which are supposed to be as annual ring. These may be subject to an accessory chek formed owing to a certain physiological rhythm of the animal.

(7) Conclusion

Within the limit of the present investigation, the following matters at least may be given as the conclusion.

(a) The relationship between the operculum length (R) and the shell-length (S) may be represented by the formula,

$$S=BR+\alpha, \quad \text{where } \alpha=1.1 \text{ mm}, \quad B=1.91 \text{ mm}.$$

(b) The value of α well agrees with the shell-length at which the operculum firstly appears.

(c) Backcalculation of the shell-length is possible by the FRESER's formula:

$$Sn=\alpha+\frac{R_n}{R}(S-\alpha)$$

(d) The age-determination of this animal is possible by means of operculum analysis.

(e) As the annuli of the operculum are thought to be formed during the winter season, the first annulus (r_1' or r_1) is supposed to be achieved at half year after hatching, and the second annulus at one and half years after hatching.

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* Also it that the formation of opercular rings correspond to hibernation has been known *Viviparus viviparus* (L.) by HUBENDICK, B. [1948. Über den Bau und des konzentrischen Operculartypus bei Gastropoden. Arkiv för Zoologi, 40A (10), 1-28].

(f) The average and the range of variation (2*SD*, given in parentheses) of the length from the focus to each unnulus of the operculum are 10.37 mm 4.37–16.01 mm) in the first unnulus and 23.0 mm (14.64–31.36 mm) in the second one.

(g) The shell-length of this animal measures 20.90 mm on average, 10.13–31.67 mm on $\pm 2SD$ at half year after hatching, and 45.03 mm on average, 29.06–60.99 mm on $\pm 2SD$ at one and half years after hatching.

(h) Two accessory resting zones (r_2 and r_3) are found to exist on the operculum during next a year of hatching.

(8) References

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ON A NEW MYXOSPORIDIAN PARASITE, *MYXOBOLUS DERMATOBIUS* N. SP., PARASITIC IN THE INTEGUMENT OF *CYPRINUS CARPIO* L.

Toshikazu Hoshina

(Received Jan. 20, 1953)

In Oct. 1938, the writer had an opportunity to study the myxosporidan-like parasite of a carp, *Cyprinus carpio*, L. by the kindness of his respected teacher the late Nobutaka Nakai. Under the microscope it was proved that the parasite was a species in the genus *Myxobolus*, myxosporida. As the parasite seems to have been hitherto unrecorded, the writer gives a description of it under the name of *Myxobolus dermatobius*.

Before proceeding, the writer wishes to thank the late Nobutaka Nakai for his kind supervision. Further he expresses his thanks to Dr. Yaichiro Okada who kindly offered him facilities for the publication of this work. His thanks are also due to Mr. Jun Suzuki the director of the Tokyo Fisheries Experimental Station for his supply of the infected carp of this parasite.

Lastly, he must express his hearty thanks to the late Makoto Koizumi for his kindness in reading the original manuscript.

Myxobolus dermatobius n. sp.

Habitat.—The parasite was found in the integument of *Cyprinus carpio* L. The fish, 25 cm long, 372 g weight, was found among the carps which on Oct., 1938, Mr. Jun Suzuki bought from the Gunma Prefectural Fisheries Experimental Station in Maebashi City for his study, and it came under the writer's observation in living state. The fish showed gray whitish small nodules in the head (Pl. VII, Fig. 1). However a few cysts were found in the trunk also.

Vegetative Form.—The cysts were lodged in the corium of the host skin and surrounded by the connective tissue of the host origin (Pl. VII, Fig. 2).

They are elliptical, measuring 1.04～1.47 mm × 0.90～1.35 mm. Along the periphery of the cyst there was a narrow homogenous cytoplasmic zone (ca. 4 μ thick) without nuclei, and on the inside of this zone the cytoplasm appeared vacuolated and dotted with numerous nuclei, spronts and developing spores at various stages, and towards the central portion, the cytoplasm were reticulated and filled with numerous spores. The sporont is mono-sporoblastic. The trophozoite is polysporous.

Spore.—Oval form in the front view, the body is gradually attenuated towards the posterior and it is elongated oval form in the side view and broad fusiform in the end view. The shell-valves are smooth, moderately thick and exhibt several markings in the front view. The sutural edge is moderately wide, and the sutural line distinct. Two polar capsules at anterior end are pyriform, equal in size and convergent. A small intercapsular appendix is present. Coiled polar filaments are seen clearly in the fresh state, coiling six

rounds, and extruded filaments are 31.0～39.0 μ long. The sporoplasm is large and has a comparatively large iodinophile vacuole, which measures 3.1～4.7 μ in diameter. When stained, the sporoplasm show two compact nuclei (Pl. VII, Figs. 3–6). The dimensions of spore are compiled in the table 1.

Table 1. Dimensions of the spore.*

Items	Nos. of spore measured	Range μ	Mean μ	S. D.
Length of spore	60	9.71～13.43	11.466±0.069	0.790±0.049
Breadth of spore	〃	7.98～10.91	9.328±0.059	0.618±0.038
Thickness of spore	〃	5.45～7.71	6.678±0.046	0.483±0.031
Ratio of length to breadth of spore	〃	1.14～1.58	1.24 ±0.010	0.108±0.007
Length of polar capsule	100	3.59～5.12	4.545±0.031	0.453±0.022
Breadth of polar capsule	〃	2.34～3.66	2.848±0.018	0.261±0.013

* The fresh spores in NEMECZEK'S hangingdrop preparation were measured by Schraubenmikrometer.

Sporogony.—As already mentioned, numerous nuclei are found in the peripheral cytoplasm of the cyst. Among those nuclei, some are found enveloped by a special condensed cytoplasm, such as mono-, two-, three- or four-nucleated bodies. The mono-nucleated body may be the sporont at the earliest stage (Pl. VII, Fig. 7). The observation, however, reveals that some of them multiply by mitotic division without the processes of their direct sporogonic development (pl. 1, Figs. 8–14). The two-, three-, or four-nucleated bodies seem to be resulted from the division without cytoplasmic fission (in this case, the mono-nucleated boy may be called schizont). The chromosomes are four in diploid. The division's fignre, however, frequently shows the two chromosomes moving toward each pole. But the figure seems to show the combined form of the two pices of chromosomes as already accepted by previous workers in the other myxosporidian species and indicate the diploid division.

By the first heteropolar division the mono-nucleated body divides into two unequal cells. These two cells remain not separating from each other and then the plasmogamy takes place and a two-nucleated body carrying a large and a small nucleus is formed (Pl. VII, Figs. 15–19). This two-nucleated bady may be the sporont at the first stage of the sporogonic development and if it develops as it is without any change, it would become mono-sporoblast and make itself a spore. Namely these two nuclei divide into two large and two small nuclei respectively. The chromosomes of the large nucleus number four. The two small nuclei divide no more, and they serve as vegetative nuclei of the sporont, and

are not directly concerned in the spore-formation. The two large nuclei continue the division and four large nuclei are formed, then the nuclear division takes place and six nuclei are formed as the result and with sporulation advancing, two differentiate for capsulogenous, two for shell-valves-forming and the remaining two for sporoplasm nuclei (some two nuclei seem to remained without dividing on the fourth nuclear division) and a spore is thus formed from these nuclei (Pl. VII, Figs. 20–25). The process of sporulation is similar to that of other myxosporidian species in the fundamental respects.

Next, the di-sporoblastic sporont seems to result from the union of the two mono-sporoblastic sporonts. In the first place the four-nucleated body with the two large and two small nuclei is formed by the union (Pl. VII, Fig. 27). The body may be the di-sporogonic pansporoblast at the first stage. As the two sporonts are found in a vacuolated peripheral part of the cyst (Pl. VII, Fig. 26), the occurrence of the union may be conceivable during their development. This four-nucleated body may be distinguished form the four-nucleated body resulted from the nuclear divison of the mono-sporoblastic sporont in the size and in the location of the two small nuclei. The later is smaller than the former and the two nuclei are not apart far from each other (see Pl. VII, Figs. 22 and 27). The writer's opinion on the origin of di-sporogonic pansporoblast agrees with that of Erdmann (1917). The further development of the pan-sporoblast is as follows: the two small nuclei may divide but not always. The observation reveals that the pansporoblast contains the two small nuclei even in the advanced stage of the development (see Pl. VII, Fig. 31). The two large nuclei produce twelve nuclei by their three times binary divisions and two spores are formed from these nuclei (Pl. VII, Figs. 26–33).

The writer thinks, with the similar thought held by Georgévitch (1935), that the sporulation of the present species is carried out in diploid state during all the periods. But the heteropolar division of the sporont was observed only once during the sporogony, different from the observation on *Myxidium* by Georgévitch *ditto*. Moreover the nuclear division of the sporoplasm nuclei was not observed in the present species.

Remarks.—Five myxosporidian species belonging to the genus *Myxobolus* have been recorded as parasites of *Cyprinus carpio* L. up to the present, viz. *M. cyprinicola*, *M. dispar*, *M. oviformis*, *M. koi* and *M. cyprini*. The present species is well distinguished from all these species. Among the known species of the genus *Myxobolns*, *M. conspicus* Kudo, 1926, indicates close affinity to the present species. In the spore of *M. conspicus*, however, the anterior portion is not broader than the posterior portion as in the present form. Moreover the spore of the former differs from the later; the thickness and the breadth of polar capsule are thinner than that of the later. Further, the present form is separable from all other related species in the dimensions of spore, in the ratio of length to breadth of spore and also in the vegetative form etc., hence, the present form deserves to be considered as a new species.

Summary

1. A myxosporidian species, *Myxobolus dermatobius* n. sp. parasiting in the integument, but more abundantly on the head of *Cyprinus carpio* L. is described in the present paper.

2. The infected carp with the myxosporidian was originated at the Gunma Prefectural Fisheries Experimental Station, Maebashi City, Gunma Pref. in Oct., 1938.

3. The brief information of sporogony of the present species is given. In the sporulation, mono- and di-sporeformation were observed. A spore may be formed from a single sporont, and two spores from a united body of the two sporonts. The sporulation is carried out in diploid stage during the entire period.

4. The present species has four chromosomes in diploid.

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PLATE VII

Fig. 1.—Showing the external view of the infected carp with *Myxobolus dermatobius*.

Fig. 2.—A cyst of *M. dermatobius* in section, parallel cut to the long-axis of the host body, stained with safranin-Delafield's haematoxylin.

Figs. 3-6.—Showing the spores of *M. dermatobius*: Figs. 3-5, front, side, and end views of fresh spores; Fig. 7, a Delafield's haematoxylin-eosin stained spore.

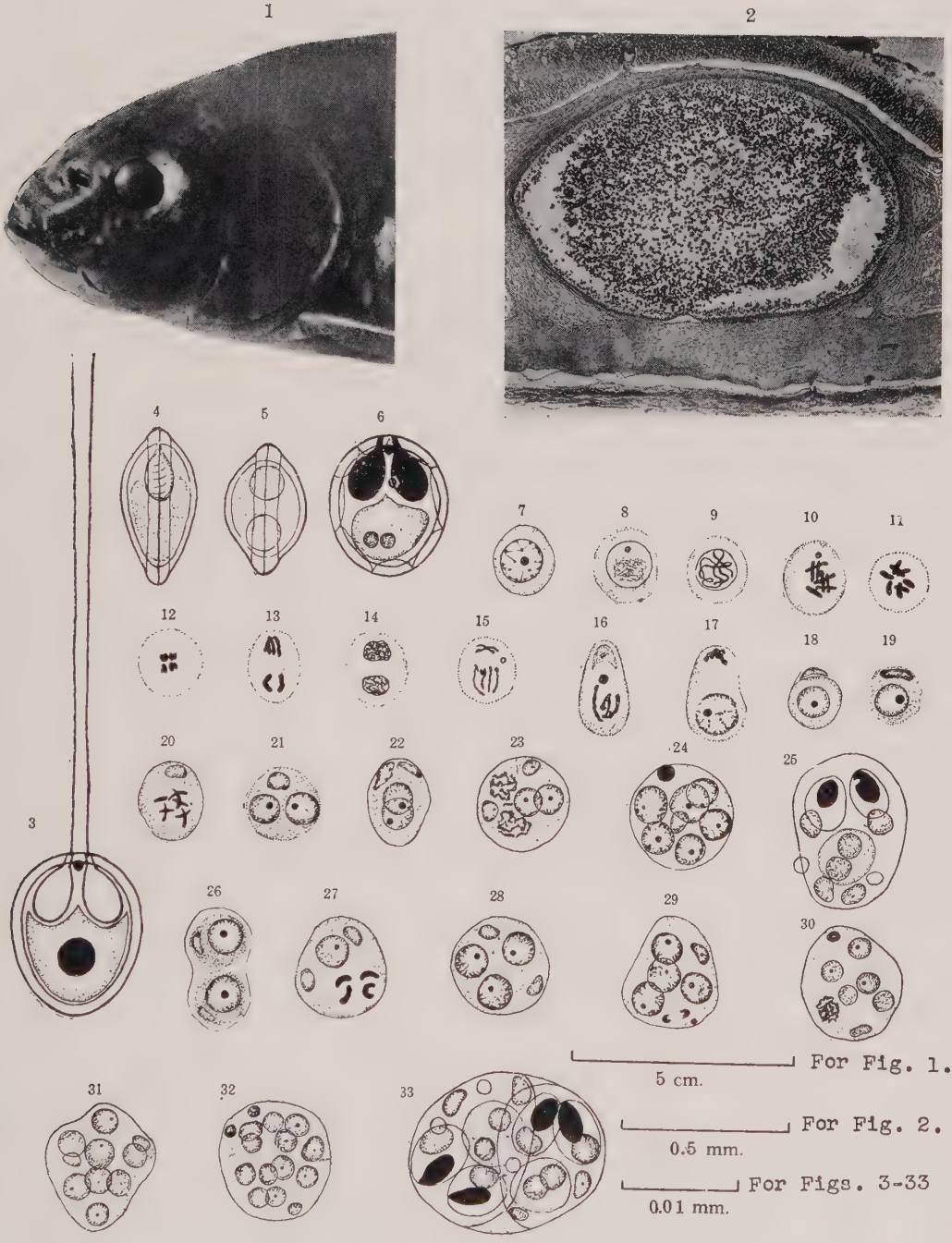
Fig. 7.—A sporont.

Figs. 8-14.—Showing the nuclear division of the sporont (or schizont): Figs. 7-11, a prophase; Fig. 12, a metaphase; Fig. 13, a anaphase; Fig. 14, a telophase.

Figs. 15-25.—The development of the mono-sporoblastic sporont: Eigs. 15-17, showing the heteropolar divisions of the sporonts; Figs. 18-20, a two-nuclear stage with a large and a small nucleus; Fig. 21, a three-nuclear stage; Fig. 22, a four-nuclear stage, with two large and two small nuclei; Fig. 23, a six-nuclear stage with four large and two small nuclei, the two large nuclei showing division; Fig. 24, a eight-nuclear stage with six large and two small nuclei; Fig. 25, a sporoblast in an advanced developmental stage.

Fig. 26.—Showing the two sporonts which are found in a peripheral vacuolated part of the cyst-

Fig. 27-33.—The development of the di-sporogonic pansporoblast; Fig. 27, a four-nuclear stage with two large and two small nuclei; this four; nucleated body may be resulted from the union of the two sporonts, and one large nucleus is under division; in the figure, two large chromosomes are seen at both poles but each of them may be equivalent to the two pices; Fig. 28, a five-nuclear stage with three large and two small nuclei; Fig, 29, a siv-nuclearstage, one small nucleus shows division; Fig. 30, a eight-nuclear stage; Fig. 31, a ten-nuclear stage; Fig. 32, a young pansporoblast with twelve large and three small nuclei; Fig. 33, a pansporoblast at in an advanced stage in development.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0.5 mm.
For Figs. 3-33
0.01 mm.

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ON THE BREEDING AND HYBRIDIZATION AMONG THE SALMONOID FISHES I.

Densaburo Inaba

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Since Fraas (1854), the hybridization among the Salmonids has been reported in many cases such as Arens (1893), Bean (1889, 1903), Day (1882, '84), Grote, Vogt et Hofer (1909), Haak (1888, '93, '94), Schindler (1938) and others. In Japan, Fujita (1926) and Koshida (1940) have been tried combined crossing of the following species, *Oncorhychus keta*, *O. masou* (both sea-run form and landlocked form) and *O. gorbuscha*, and described the characters of these hybrid larvae and young. Hiraki (1931) reported fast growth of the hybrid between *O. masou* (sea-run form)×*O. rhodurus* which had been tried since 1919 under the suggestion of Dr. C. Ishikawa, and Watanabe (1924～'26) showed the same fact in the pond culture. Soguri (1931) reported for the first time the crossing among foreign and Japanese species, i.e. Japanese charr (*Salvelinus pluvius*)×American brook trout (*S. fontinalis*), *S. pluvius*×*O. masou* (landlocked form) and *O. masou* (sea-run form)×*S. fontinalis*. He reared these hybrids for two years or more and reported the characters of adult form of hybrid between *S. pluvius*×*S. fontinalis*. Dr. K. Kuronuma tried in 1944 the crossing between Brown trout (*Salmo trutta*)*×*O. keta* but all the materials and notes were burned by fire in time of War.

In our mountain rivers and streams the artifical propagation of native trouts, such as *O. masou* and *O. rhodurus*, has many difficulties to obtain sufficient numbers of the adults, especially female, but the eggs of *S. fontinalis* are obtained by rearing so easily that I tried the hybridization among *S. fontinalis*, *O. masou* and *O. rhodurus* in the breeding season of 1943 and 1944. These hybrids have been reared at Ôizumi Trout Hatchery of our University, and since autum, 1950 they had shown their sexual characters. The results and the course of rearing may be given in the following pages.

Before going further I wish to express my hearty thanks to Mr. Z. Uematsu for his rearing of fishes and also to Mr. M. Nomura for his kind help on laboratry works. Also indebted to Dr. K. Kuronuma for his helpful suggestion and correction of the original manuscript.

1. Hybrid between *Salvelinus fontinalis* (♀) and *Oncorhynchus masou* (♂)

On December 3, 1943 and on November 30, 1944, for each case, about 1700 eggs of American brook trout (*S. fontinalis*) were fertilized with milt of

* The writer adopts the name *Salmo trutta* for Brown trout found in Japan regardless of the nomenclatorial confusion for the species.

O. masou which had been transported in eyed ova from Aisaka, Aomori Prefecture in 1940 and have been reared at Ôizumi Trout Hatchery since. The parent fishes used, are as follows.

Table 1. Measurements of parent fishes which used for experiment.

year of experiment	*S. fontinalis*			*O. masou*		
	age	Body, Length	Weight	age	Body, Length	Weight
		cm	g		cm	g
1943	3	24.5	280	3	21.3	185
1944	3	22.5	265	4	22.5	210

These eggs hatched on January 15, 1944 and January 13, 1945, in 43 and 44 days, respectively.** On February 25, 1944 and on February 20, 1945, the fry begun to feed and grew about 2.0 cm in length, 0.08 g in weight, but the remainders reduced to 260 and 340, respectively. One year after, reared in pond, the young grew about 8.7 cm in length and 9.5 g in weight, and the number were counted 231 and 153 respectively. At the end of November 1946, total alived fry were reduced to 51 with the length of about 18 cm. Until two years after, ellipsoid Paar-marks were remained counting 7 to 11 on the both sides of body.

Table 2. Measurement of adult hydrid between *S. fontinalis* and *O. masou* reared at Ôizumi Trout Hatchery.

Sex	Total Length	Body, Length	Body, Depth	B.L. B.D.	Head, Length	B.L. H.L.	Snout, Length	Body, Width	Eye, Diameter	Weight
	cm	cm	cm		cm		cm	cm	cm	kg
Female	47.0	42.5	10.5	4.04	9.8	4.33	2.8	5.5	1.6	1.10
〃	37.8	34.0	8.5	4.00	8.5	4.00	2.5	4.2	1.7	0.78
〃	37.7	33.1	8.1	4.08	7.9	4.18	2.2	4.2	1.3	0.60
〃	37.5	33.5	7.8	4.29	7.5	4.46	2.3	4.5	1.4	0.62
〃	37.5	33.5	8.0	4.18	8.3	4.03	2.4	3.8	1.5	0.57
〃	36.3	32.8	8.3	3.95	8.5	3.85	2.3	3.8	1.3	0.57
Mean	38.96	34.90	8.53	4.09	8.41	4.14	2.41	4.33	1.46	0.706
Male	38.0	34.0	10.2	3.26	9.5	3.57	3.0	4.3	1.4	0.65
〃	37.8	33.9	9.9	3.42	9.1	3.66	2.8	4.3	1.4	0.72
Mean	37.90	33.95	10.05	3.34	9.30	3.61	2.90	4.30	1.40	0.685

The slow growth and high mortality above mentioned were thought chiefly due to the lack of food during the War time. Then the food supply became gradually abundant, 1950, having eight fishes remained alive, at the end of November which showed their sexual characters. Six of remainders were females and two were males (Plate. 1. figs. A, B). The male slightly humbacked and had strongly developed jaw, long snout and produced a trace of milt. The female was slender in shape and sterile as SOGURI[12] reported in the reciprocal hybrid. Their dimensions of these hybrids are shown in the following table (Table 2). Three of them are alive still as of January 15, 1953 and the rearing is continued in pond.

**Water temperature Ôizumi Trout Hatchery is constantly 10.6±0.1°C. Eggs of *S. fontinalis* usually hatch within 42-45 days.

The general characters of this hybrid are as follows. Dorsal fin rays 11～13; Anal fin rays 11～13; Branchiostegales 11～13; Gillraker 17～18; Scales on lateral line 120～126; Pyroric coeca about 53; Teeth on vomer in one row, poorly developed.

Colour is yellowish brown with wavy black streaks as seen on the back side of *S. fontinalis* but they are more slender and spread to the ventral portion beyond lateral line. No red spots on the both sides of body and no white margin on each front edge of pectral, ventral and anal fin. On dorsal and candal fin there are no wavy black streaks but with small black spots. In the breeding season the ventral portion of the male turn to reddish brown as in *S. fontinalis*. Scales are round deviating from that of *S. fontinalis* which show small elongated ellipse (fig. 1, A), and most of the outer circuli detach on their

Fig. 1. A. Scale of *Salvelinus fontinalis*. ×50. B. Scale of hybrid, *Salvelinus fontinalis* (♀)×*Oncorhynchus masou* (♂). ×20. C. Scale of hybrid, *Salvelinus fontinalis* (♀)×*Oncorhynchus rhodurus* (♂), ×20.

exposed area with 10 to 12 continious circuli seen near the focus (fig. 1, B). They have the measurment of about 1.6 mm long and 1.7 mm wide, but in the latter they measure about 0.9 mm and 0.5 mm. These measurements are shown in Table 4.

In external chardeters this hybrid generally resemble *O. masou* except its wavy streaks and colouration during breeding season.

2. Hybrid between *Salvelinus fontinalis* (♀) and *Oncorhynchus rhodurus* (♂)

On December 2, 1943 about 1500 eggs of *S. fontinalis* were fertilized with the milt of *O. rhodurus* which transported in eyed ova from Nikko, Tochigi Prefecture in 1940 and have been reard at Ôizumi Trout Hatchery since. In 1944, the same hybridization was repeated but resulted in vain owing to death of fertilized eggs. The parental fish of *S. fontinalis* used was in 3rd age, 22.3 cm in body length, 275 g in weight and that of *O. rhodurus* was in 3rd age, 18.2 cm in body length, 65 g in weight.

These eggs hatched on January 16, 1944 or in 46 days. On February 25, they begun to feed and alived fish which grown about 2 cm, 0.08 g, were about 500 in number. These fry have been reared in the same poor conditions as the former hybrid so that they also show high mortality and slow growth. They have grown about 8 cm in total length, 9 g in weight at the end of the year. In October, 1945, the remainders reduced to 140 in number (about 14 cm, 17 g) and in October, 1947, they counted 26 (about 22 cm, 75 g). Parr-marks of round elliposid counting 7 to 8 and remained during that time. At the end of November, 1950, the total remainders were 7 which showed sexual characters. Three of which were females and four were males (Plate 2, figs. A, B). The adult forms of this hybrid show smaller size than the former hybrid but larger than river form of *O. rhodurus*. The male is not sterile same as the former and has strongly hooked lower jaw and elongated snout. The female was not sterile but had a few normal eggs (4 to 15), thus the fertilization prooved vain. Their dimensions were measured in Table 3. Four of them are still alive as of January 15, 1953 and the rearing is continued in pond.

Table 3. Measurement of adult hydrid between *S. fontiulis* and *O. rhodurus* reared at Ôizumi Trout Hatchery.

Sex	Total Length	Body, Length	Body, Depth	B.L./B.D.	Head, Length	B.L./B.D.	Snout, Length	Body Width	Eye, Diameter	Weight
	cm	cm	cm		cm		cm	cm	cm	kg
Female	36.0	31.0	7.0	4.42	7.5	4.15	2.7	3.7	1.2	0.53
〃	33.5	30.5	7.5	4.06	7.5	4.06	2.0	4.3	1.4	0.43
〃	32.8	29.7	6.8	4.37	7.1	4.16	2.1	3.2	1.2	0.42
Mean	34.10	30.40	7.10	4.28	7.37	4.13	2.26	3.73	1.26	0.462
Male	38.5	32.5	9.3	3.49	9.3	3.49	2.9	3.8	1.4	0.59
〃	33.7	28.0	7.0	4.00	8.2	3.41	2.5	3.4	1.4	0.36
〃	28.8	24.0	6.8	3.53	7.2	3.33	2.3	2.8	1.9	0.26
〃	27.5	24.5	7.3	3.32	7.8	3.11	2.5	3.5	1.4	0.31
Mean	31.87	27.20	7.40	3.58	8.12	3.33	2.55	3.37	1.52	0.380

The general characters of this hybrid are as follows. Dorsal fin rays 12; Anal fin rays 10～11; Branchiostegales 10～12; Gill-rakers 18～19; Scales on lateral line 120～125; Pyloric coeca about 37; Teeth on vomer only in its head and poorly developed.

Most of colouration was same as the former hybrid but wavy black streaks of body and black spots on dorsal and caudal fins were broader than that of the former. No red spots exist, which are possessed by both parent forms. In the breeding season, the colour of ventral portion turned to reddish brown similar to that of *S. fontinalis* and the former hybrid.

Scales were round ellipsoid (fig. 1, D) and their diameters were about 2 mm long and 1.6 mm wide, the measurements shown in Table 4.

In external characters, the adult form of this hybrid generally seems to be closer to *O. rhodurus* than *S. fontinalis*.

Table 4. Measurements of scales in the three forms of trouts, reared at Ôizumi Trout Hatchery.

Salvelinus fontinalis		*S. fontinalis* × *O. masuo*		*S. fontinalis* × *O. rhodurus*	
Length	Width	Length	Width	Length	Width
mm	mm	mm	mm	mm	mm
0.70	0.37	1.48	1.82	1.86	1.44
0.69	0.33	1.26	1.70	1.76	1.58
0.82	0.39	1.90	1.80	1.76	1.42
0.96	0.50	1.44	1.60	2.02	1.66
0.96	0.40	1.78	1.92	2.04	1.64
0.94	0.40	1.82	1.82	2.12	1.80
1.12	0.56	1.64	1.56	1.94	1.48
1.10	0.66	1.48	1.48	2.28	1.80
0.84	0.76	1.64	1.76	2.00	1.76
0.92	0.56	1.50	1.70	2.14	1.80
Mean 0.885	0.489	1.594	1.716	1.992	1.638
Mean L/W	1.867		0.928		1.219

3. Discussion

The occurrence of red spots on sides is an important characters to identify the river forms of *O. rhodurus* from *O. masou*. The above mentioned two types of hybrids have no any red spots but have the wavy black streaks. So, the differences seen in the features of the streaks become an important point for the discrimination of two hybrids. The streakes of the latter are broader than the former. Soguri[12] reported the same colouration in the hybrid between *O. masou* (♀) and *S. fontinalis* (♂). Similar configuration of streaks are also reported in the hybrids among *S. fontinalis* and *Salmo levenensis*[3,4], *S. salvelinus*[11], *S. trutta*[2,3,4,6,7]*, and they are known as Zebra, Leopard hybrid or "Tigerfisch", which some times found even in natural waters in America[2] and in Europe[6]. The development of the "vermiculation" (wavy dark streaks on the lighter back ground) seem to weaken by oder owing to their bigness of spots on sides; hybrid between *S. fontinalis* and *Salmo trutta*, that of *O. rhodurus*, that of *O. masou*. Such a colouration of *S. fontinalis* is interpreted to be inherited dominantly in the above mentioned cases of hybridization.

There are, however, the intermediate type of parental colouration such as in the hybrids between *S. fontinalis* and *S. struanensis*, that of *S. alpinus*[3,4] as well as that of *S. pluvius*[12]. These are the cases so called Struan hybrid which have poor wavy black streaks and both red and white spots occurring on sides of body. These differences in colouration among hybrids, seems to me, may give a suggestion for the discussion in the relationship of *S. fontinalis*, and the former cases seems to show the remote relation than the latters.

The white margin on each front edge of anal, pectral and ventral fin disappear in my two hybrids but it remaine clearly in Zebra hybrid, Struan hybrid and that of *S. pluivus*. Judging from these facts, the occurrence of white

* These authors use the scientific name of *Salmo fario*, *Trutta fario* or *T. trutta*.

margin on the fins in the hybrids seem to show close relationship to *S. fontinalis*.

Red spots on sides might be a significant character in some cases, but their appearance or disappearance in hybrids seems to have only a secondary meaning to interprete the relationship of salmonoid fishes.

The other characters such as number of dorsal fin rays, anal fin rays, branchiostegals, gill-rakers and others show some differences between two hybrids which I experimented. These characters of two hybrids compared with those of parent forms are summerized in Table 5.

Table 5. Characters of the hydrids and parent fishes.

Species / Character	*Oncohrynchus masou* (♂)	Hydrid *S. fontinalis* × *C. masou*	*Salvelinus fontinalis* (♀)	Hybrid *S. fontinalis* × *O. rhodurus*	*O. rhodurus* (♂)
Dorsal fin rays	12–16	11–13 (13–14)*	9–10	12	13–15
Anal fin rays	14–18	11–13 (11–15)	8–9	10–11	12–14
Branchiostegals	10–13	11–13	10–12	10–12	11–12
Gill-rakers	18–19	17–18	17	18–19	17–20
Scales on lateral line	130–140	120–126	122–205**	120–125	124–133
Parr-marks	6–9	7–11 (7–11)	7–10	7–8	7–8
Pyloric coeca	40–55	about 53	28–36	about 37	42–76
Pattern on sides	black small spots	wavy black streaks, slender	wavy black streaks and black spots	broader w.b.st.	black spots
Red spots	none	none	exist	none	exist

* number in parenthesis show the results of SOGURI[12]; ** 230 are given in many text-book, but I cannot find this number in my samples. DAY[3] states the same fact.

The bearing of the present hybryidization experiment to the fish conservation is incidentally arrived as follow. The river form of *O. masou* and *O. rhodurus* is conserved in the natural water by means of some protective measures instead of stocking of hatching raised fingerings at least in the present time, becomes of the difficulties derived from artificial egg taking at hatchery. Under such circumstances it may be resonately suggested that the hybrid such as I tested but with an easy procedure in our hatcheries is produced in bulk and stocked in streams, it is permitted to presume the hybrid characterized by higher adaptation to our native streams than for American brook trout, the female of which being abundantly obatinable in our trout hatcheries.

Summary

The hybridization between *S. fontinalis* and *O. masou*, and between the former and *O. rhodurus* was carried out and the hybrids obtained have been reared since 1944 at Ôizumi Trout Hachery of our University. The results are

summarized as follows.

1. The two hybrids grow about 40 cm in length and they are larger than the river form of *O. masou* and *O. rhodurus* which are about 20 cm. They are alive 8 years or more in rearing pond.

2. The wavy black streaks as in *S. fontinalis* are inherited dominantly and they spread downward to ventral portion of body. Nuptial colour in the breeding season appears same as in *S. fontinalis*. The other characters resemble the father fish *O. masou* or *O. rhodurus*.

3. Red spots on sides and white margin on the front edges of fins disappear in the two hybrids. The wavy black streaks in the hybrid orignated from *O. rhodurus* are broader than that from *O. masou*.

4. Scales of two hybrids are round on the contrary to ellipsoid found in that of *S. fontinalis*, but with few (10 to 12) continuous circuli on the exposed area.

5. The male in the two hybrids a little milt. The female of *O. masou* hybrid is sterile, but that of *O. rhodurus* has a few eggs.

6. The releasing of these hybrids fry may be a procedure for the maintenance of the river trout, becouse of their closer adaptability to the native enviroment and easiness of obtainning fry or fingerling in hatcheries.

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PLATE VIII.

Hybrid between *Salvelinus fontinalis* (♀) and *Oncorhynchus masou* (♂).

A. Adult male. 33.9 cm in body length.

B. Adult female. 33.1 cm in body length.

PLATE IX.

Hybrid between *Salvelinus fontinalis* (♀) and *Oncorhynchus rhodurus* (♂).

A. Adult male. 24.0 cm in body length.

B. Adult female. 29.7 cm in body length.

A
B
0
5 cm.

A
B
0
5 cm

Journal of the Tokyo University Fisheries, Vol. 39, No. 2, 1953

STUDY ON THE DISPOSITION OF FISH TOWARDS THE LIGHT. REPORT NO. 1 STUDY ON THE BEHAVIOUR OF *TRACHURUS JAPONICUS* TO THE LIGHT

Yutaka IMAMURA

(Received Jan. 15, 1953)

Introduction

Generally speaking, the fish gathering around the light are divided into two types : some fish gather around the light directly, and the others indirectly. In the fishing that uses the light, it is an important matter to know in what way, either directly or indirectly the fish gather around the light. In the case of the study on the fishing which uses the light, it is necessary to conclude the next two problems.

Problem,

No. 1. Study on the behaviour of fish at night.

No. 2. Study on the disposition of fish towards the light ..... the fish gathers either directly or indirectly around the light.

This experimentation was practised with the horizontal directional submarine light, 60 watt, at the pier in TATEYAMA-Bay. Water-temperature 25.5°C, Date 18th-19th, Aug. 1952.

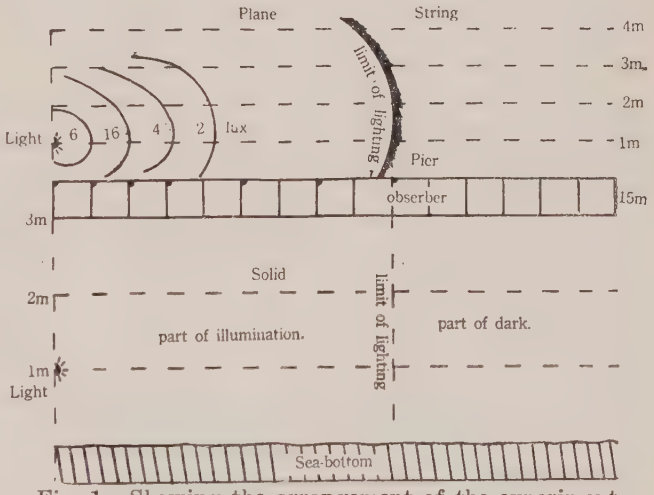

Fig. 1. Showing the arrangement of the experiment apparatus.

§ Experiment No. 1.

Place·····At the pier in TATEYAMA-Bay (belonging to Tokyo University of fisheries) the age of the moon 29th. Experimental-facilities.....See the fig. No. 1. The source of light.....horizontal directional submarine light 60 watt. The *Trachurus japonicus*, the *charbdea rastonii hoacke* and the plankton was observed by 7 observers.

General conditions of this experiment.

8h-30 m. Lighting.

8h-10 m. Saw the planktons, they assembled towards the light immediately after lighting. The *Charbdea rastonii hoackes* appeared on the water surface near the light.

9h-10 m. Saw a few *Trachurus japonicus*, which were swimming in part of limits the illumination. The fish form groups.

9h-30 m. The *Trachurus japonicus* massed in a small circle, after a while, the shoal slowly approached towards the light along the sea bottom. The shoal grew larger and larger as new-comers joind. The planktons formed a large group around the source of light. They assembled directly towards the light by spiral-movement. The *Charbdea rastonii hoackes* assembled on the surface of the water. They were about 80–100 in number. They caught planktons and swarms which were assembling towards the light. That is to say, they assembled indirectly towards the light.

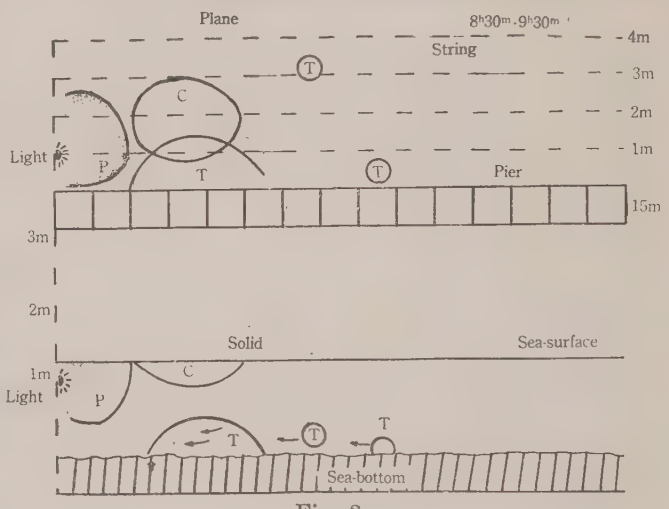

Fig. 2.

10h-30 m. The group (about 100 fishes) of *Trachurus japonicus* began to rise to the surface from the sea bottom, then the group approached the light. But the group did not make straight for the light. The group moved from the part of lower illumination to that of higher. The planktons formed a white wall in the bundle of the light. The *Charbdea rastonii hoackes* were distributed near the source of light, while the planktons caught the insects and ate them.

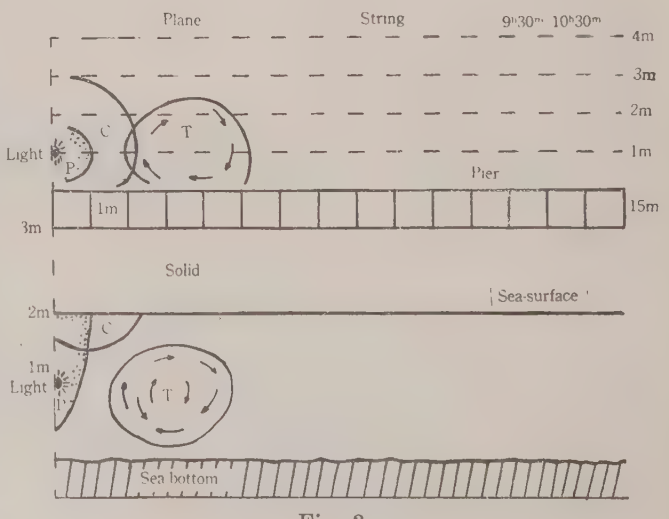

Fig. 3.

11h-30 m. The group of *Trachurus japonicus* was disordred, the density of fishes became thin. The fish moved freely up and down, left and right, towards the

lighting zone. The *Trachurus japonicus* stayed in a part of higher illumination during 30 second–1 minute, then they left for a part of lower illumination. The fish stayed in a part of lower illumination during 1-2 minute, and sought for baites. While the fish swam towards the light again. This movement of the fishes was reapted more than once.

12h-30 m. The swimming-fish arrived at the part of limits illumination. The group of fishes separated into few small group (5–10 fishes) or remained single. And then, they sought for baits actively. The *Charbdea rastonii hoacke* assembled about two hundreds and their movement towards the light was active.

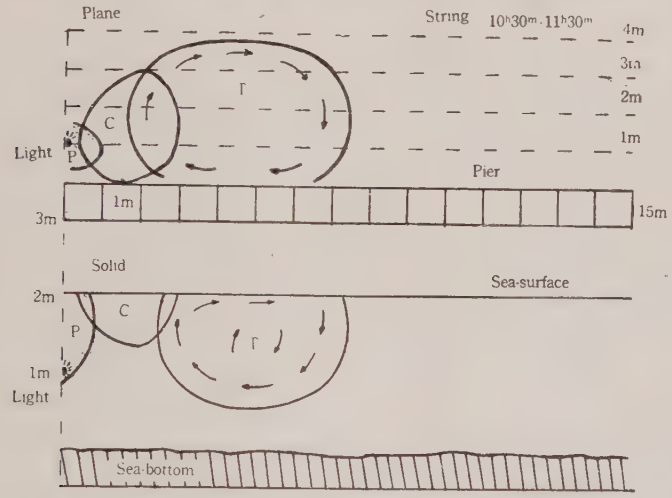

Fig. 4.

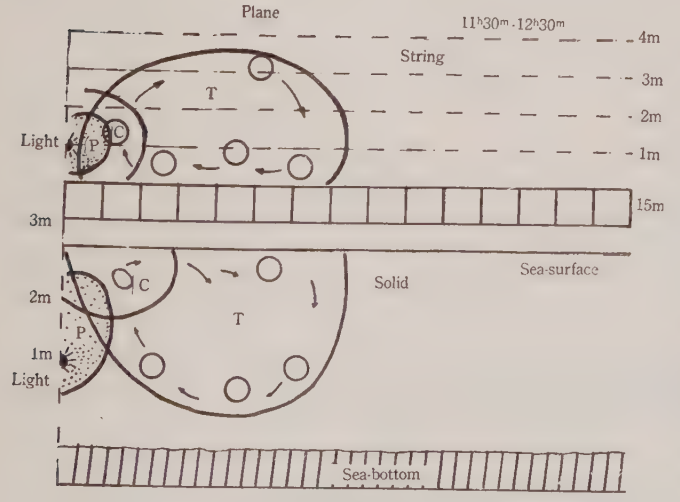

Fig. 5.

Fig. 2-5. Showing the fish gathered towards the light.
P.....plankton. C.....*Charbdea rasnonii hoacke*
T.....*Trachurus japonicus.*

1h-30 m. The movement of *Trachurus japonicus* was similar as above.

Result of Experiment No. 1.

(1) The action of planktons towards the light was instinctive. This is the direct action.

(2) The *Trachurus japonicus* swam single or in a group of 2–3 fish in the dark night.

(3) The *Trachurus japonicus* formed a great shoal in a part of the illumination.

(4) The fish appeared in a part of illumination, stayed a few minute in the part of illumination. But they did not swim straight to the light. The movement of the fish in the part of lower illumination was slow. This state of fish is a good chance for the net fishing.

§ Experiment No. 2.

Measurement of gathering-speed, *Trachurus japonicus* towards the light. This measurement was practised for 128 fishes.
No. 1....in lighting.

(a) The *Trachurus japonicu's* shoal (128 in number) was drived out of the illumination circle with a pole. Was measured the time until they resumed to form a shoal (100 fishes) in the illumination circle.

Table 1.

Time of threat.	40 s	1 m-0 s	2 m-0 s	3 m-0 s	4 m-0 s	4 m-0 s
Time until they to form a shoal	1 m-20 s	1 m-40 s	2 m-30 s	3 m-50 s	4 m-20 s	4 m-40 s

The time until they ressumed to form a shoal is in proportion to the time of threat.
No. 2.....This experiment was practised alternately during lighting and black-out.
notice....This experiment was practised to the shoal (*Trachurus japonicu's*) which is accustomed to the light.

(a) In the case of the momentary black out (1 second) *Trachurus japonicu's* shoal (124 fises) was unchanged.

(b) ——....lighting. (1 minute) ×....black-out (1 second)

— × — × — × — × — × — × — × — × — × — × —
No. 1 No. 2 No. 3 No. 4 No. 5 No. 6 No. 7 No. 8 No. 9 No. 10

The *Trachurus japonicu's* shoal (124 fishes) was unchanged until No. 3. The shoal was divided into a few small group from no. 4 and stopped to eat baits and was impatient. But the fish did not run away from the circle of illumination.

(c) ——....lighting. ×....black-out (30 seconds)

——×——

The group of planktons or *Charbdea rastonii hoacke* was unchanged. The *Trachurus japonicu's* shoal (106 fishes) dispersed. There were found 48 fishes 1 minute after lighting. Found 96 fishes 8 minute after lighting. The fishes began to eat 15 minute after lighting. The 10 fishes ran away.

(d) ——....lighting. ×....black-out (5 minute)

——×——

The *Trachurus japonicu's* shoal (96 fishes) dispersed perfectly. There were found a few fishes 3 m after lighting. 4 m....22 fishes. 6 m....46 fishes. 18 m....68 fishes. 20 m....74 fishes.

22 fishes ran away. The group of planktons dispersed and the density of planktons was thin. But lighting again, the density of planktons increased 1/3 1 minute after. After 3 m the planktons returned to normal. The *Charbdea rastonii hoacke's* group dispersed. 2 minute after
lighting again....1/3 8 m....returned to normal.

Results of Experiment No. 2

notice....This experiment was practised to the *Trachurus japonicus* shoal.

(a) (more than 80 fishes).

Tabie 2. A, Time until more 10 fishes appeared; B, Time of the black-out.

A	2 m	3 m	4 m	4 m	7 m	8 m	11 m	8 m	13 m	12 m
B	1 m	2 m	2 m	5 m	5 m	8 m	10 m	10 m	15 m	15 m

Reciprocity between A and B.....r=0.96 (Fig. 7)

(b) The *Trachurus japonicu's* shoal which was gathering towards light dispersed in consequence of putting out the light. Generally, *Trachurus japonicus* does not form a shoal in the dark-night.

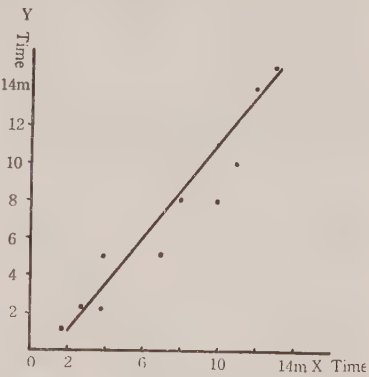

Fig. 7. Relation between the time until more 10 fishes appeared (X axis) and the time of black-out (Y axis).

§ **Experiment No. 3**

Relation between the *Trachurus japonicus* and *Charbdea rastonii hoacke* that assembled towards the light. see Fig. 6.

notice....The number of *Charbdea rastonii hoackes* was counted in the small quadrilateral circle. The number of *Trachurus japonicus* was counted in the large quadrilateral circle.

Method of experiment. The auther found a relation between the number of fishes and *Charbdea rastonii hoacke* that gathered towards the light. The two submarine light were used and they could be freely lighted or put out at the same time.

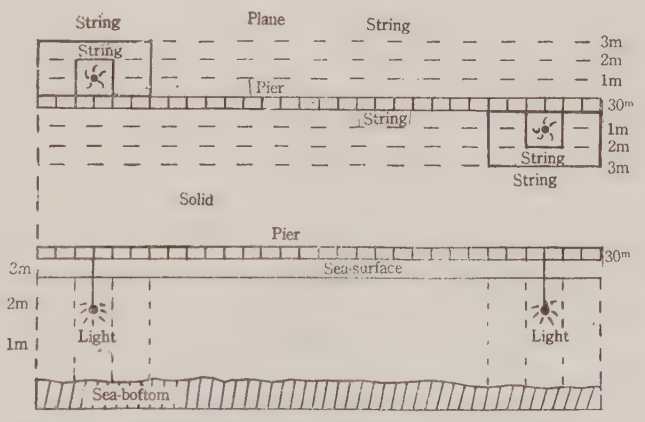

Fig. 6. Showing the arrangement of the experiment appratus.

Result of Experiment No. 3

Table 3. C, number of *Charbdea rastonni hoack*; D, number of *Trachurus japonicus*

C	15	27	38	39	42	50	60	72	78	80	80	90	100	109	112	120	140	158	159
D	4	8	12	15	19	30	18	25	21	30	25	45	32	36	40	46	61	60	62

Reciprocity between C and D....r=0.95 (Fig. 8).

§ Experiment No. 4.

Comparative test was practised between the baits and the light using scattering-bait of fish meat for the fishes gathered towards the light. The results taken are given in the following tables, 4–7. The percentages of gathering for bait are about 91% in the case of experiment (a) (Table 4), ca 100% in the cases of experiments (b) and (c) (Table 5, 6) and 0% in the case of experiment (d) (Table 7) respectively.

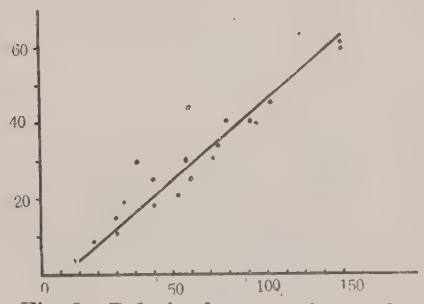

Fig. 8. Relation between the number of *Trachurus japonicus* (Y axis) and *Charbdea rastonii hoacke* (X axis) gathered towards the light.

Table 4.

E	1 m	6 m	8 m	12 m
F	18	39	68	79

Table 5.

E	30 s	1 m	2 m
F	38	65	78

Table 6.

E	5 s	15 s	1 m
F	25	74	100

Table 7.

E	5 m	10 m	15 m
F	0	0	0

Experiment (a) E....Gathering time to baits. F....number of gathered fishes.

Scattered baits in 10 meters from the *Trachurus japonicu's* shoal consists of about 86 fishes. (Table 4.)

Experiment (b) Scattered baits in 5 meters from the shoal (about 78 fishes). (Table 5.)

Experiment (c) Scattered baits in 2 meters from the shoal (about 102 fishes). (Table 6.)

Experiment (d) Scattered baits in 1 meters from the shoal (about 97 fishes). But the baits placed behind the light. (Table 7.)

§ Experiment No. 5.

Measurement of the swimming speed of *Trachurus japonicus* that gathered towards the light. notice....The fishes accustomed to the light, that is to say, the fish that is swimming parallel to the pier, we measured the swimming time per 1 meter. The data obtained from this experiment are shown in the Table 8. The average speed of this fish is found to be 7 second per 10 meters, but the momentary speed going to baits is greater than the average one.

Table 8.

Time per 10 meters	10 s	6 s	2 s	8 s	12 s	5 s	4 s
Number of measured fishes.	4	2	1	3	2	4	3

§ Conclusion and suggestion.

It is difficult to conclude that the disposition of *Trachurus japonicus* towards the light is direct or indirect. From the result of this experiment, the author has known the next facts.

(1) *Trachurus japonicus* has the power which takes advantage of light as a means of living. (2) *Trachurus japonicus* takes advantage of light as a means of making a shoal. At night, the fishes that gathered towards the light appeared single or 2–5 in number. The fish form a shoal in the part of illumination. When the light is put out, fishes of this shoal dispersed. (3) *Trachurus japonicus* takes advantage of the light so as to increase the hunting-power for baits. The auther found that the number of fishes gathered towards the light is in proportion to the number of *Charbdea rastonii hoackes* gathered towards the light. (4) Angling of *Trachurus japonicus* is practised in the daytime, but it is impossible at night, while it is possible at night with lighting. (5) Behaviour toward the light.

(a) In lighting zone, *Trachurus japonicus* appeared in a part of lower illumination at first, stayed there for a while until they a adopted themselves to that lux, and slowly approached to the part of high illumination.

Inclination towards the light *Trachurns japonicus*<*Charbdea rastonii hoacke*<plankton. *Trachurus japonicus*, inclination towards the light<inclination, towards the bait.

Conclusively the following suggestions may be afforded for the fishing by lighting, viz. (a) It is neccessary to select the place where natural baits are abundant as the fishing ground. (b) The number of fishes gathering towards the light will be increase according to the expansion of the illumination zone. The moving light is mors efiective than the fixed light. (c) The fishes which gather in a very wide illumination zone must gather again in a small circle from there.

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A STUDY ON THE SCALE OF THE GOBIID FISHES OF JAPAN

Kazunori TAKAGI

(Received January 24, 1953)

The gobiid fiishes are very common in Japanese waters, inland, coastally and sometimes off-shore, and very abundant in species, so they did interest for a long time not a few research workers. However, the workers did not seem to pay so much attention yet to the scale, while it presents various appearance according as the abundance of this family, which interests the writer to undertake the present study. As for the scales of the gobiids, at any rate, there has been in the descriptions of the fishes none but scale calculation or simple notes. Or else, some authors, as ISHIKAWA and NAKAMURA (1940)[1] in *G. brunneus*, pursue them in the course of their early development. Others notice them, analysing their characters for the purpose of the age determination of the fish. This is the case of MIYAZAKI (1940)[2] and NAKAMURA (1944)[3] in *A. flavimanus* and *C. urotaenia* respectively. KOBAYASHI (1952)[4] suggests diagramatically the genealogical tree of the gobiids based on the scale characters, but does not give a detail yet. It is interesting that FAGE (1915, p. 35)[5] adopts the scale shape as a systematic criterion for the mediterranian eleotridids, though not to concern with the Japanese species in this case.

The present writer studies comparatively on the configuration of the scales of our 21 gobies, and after making possible scrutinies gets some advanced knowledges, hoping that contribute to furnish a fundamental for the fishery biology.

The writer wishes to express his sincere appreciation to Professor Dr. I. KUBO of the university, under whose direction and encouragement the present study was initiated and accomplished, and by whose suggestions, corrections have been made in preparing this manuscript. Special thanks are also due to Dr. K. MATSUBARA, for his showing favour to the writer in various ways. The writer offers his gratitude to Ass. Prof. J. SENÔ, Technician Dr. T. INO (formerly Assistant Professor of the university), Messrs. M. NAKAMURA, S. ITO,

1. ISHIKAWA, M. and NAKAMURA, N., 1940. [Life-history of the Japanese gobiid fish, *Glossogobius brunneus* (TEMMINCK et SCHLEGEL) (in Japanese).] Bull. Sci. Fish. Ass., 8(1), 1-16, figs. 3, pls. 2.

2. MIYAZAKI, I., 1940. Studies on the Japanese common goby, *Acanthogobius flavimanus* (TEMMINCK et SCHLEGEL) (in Japanese). Bull. Jap. Soc. Sci. Fish., 9(4), 159-180, figs. 13.

3. NAKAMURA, K., 1944. [Ecological studies on the gobiid fishes. I. On the growth and the age of *Chaenogobius annularis urotaenia* (HILGENDORF) (in Japanese).] Journ. Fish., 52, 23-30, figs. 4.

4. KOBAYASHI, H., 1952. Phylogeny and evolution of Japanese freshwater fishes based on the comparative morphology of scale characters (Preliminary report) (in Japanese). Aichi Gakugeidai. Kempo., 1, 1-4, figs. 4.

5. FAGE, L., 1915. Remarques sur l'évolution des gobiidae, accompagnées d'un synopsis des espèces européennes. Arch. Zool. Expér. Génér., 55, Notes et revue (2), 17-34.

I. KUNIMINE, and Y. MATSUMOTO, for the help of his collecting the specimens.

Material and Methods

The specimens here examined are referred to 12 genera, 15 species, and 8 formae. In these 23 the large part of our common gobies are embrased, notwithstanding they are one fourth of the gobiids in Japan, which has been known after TOMIYAMA (1936)[6] to consist of 35 genera, 56 species, and 26 formae.

Table 1. Scientific name, total length (T.L., mm), date and locality of the material.

Species	T. L.	Date	Locality
1. *E. abax abax*	41.7	'47-V-13	Uchiura Bay
	44.0	〃	〃
2. *V. hanae*	96.3	———	Tateyama Bay
3. *L. guttatus guttatus*	52.0	'47-V-13	Uchiura Bay
4. *A. lacticella lacticella*	44.6	'35-IV-8	〃
	49.0	———	———
5. *A. lacticella cosmurus*	42.4	'47-VII-20	Tayu Point
	38.1	〃	〃
6. *E. gilli*	38.5	———	Haneda
7. *Z. farcimen*	37.1	'34-VII	Uchiura Bay
	32.6	'35-IV-8	〃
8. *B. fuscus*	48.2	'47-V-13	〃
	56.9	〃	〃
	62.5	'34-VII	〃
9. *R. gymnauchen*	76.2	'47-VII-19	Tayu Point
	72.7	〃	〃
10. *R. giurinus*	62.0	'48-IX-1	Uchiura Bay
	79.3	'47-VIII-17	Izumi-tamagawa
11. *A. ornatus campbelli*	94.7	———	Uchiura Bay
12. *M. abei*	32.2	'47-VII-6	Ara River
13. *O. acutipennis*	58.7	'47-VII-19	Tayu Point
14. *A. lactipes*	44.0	'47-VIII-14	Hirasaku River
15. *A. flavimanus*	122.9	'47-VIII-8	Haneda
16. *P. elapoides zonoleucus*	42.8	'34-VII	Uchiura Bay
17. *P. elapoides elapoides*	67.2	'47-V-13	〃
	79.0	'34-VII	〃
18. *P. zacalles*	138.8	———	〃
19. *C. dolichogunathus*	64.5	'34-VII	〃
20. *C. gulosus*	98.1	'47-V-13	〃
21. *S. geneionema*	90.5	'35-IV-8	〃
22. *T. obscurus obscurus*	88.3	'34-VII	〃
23. *T. trigonocephalus*	61.5	'34-VIII-10	———

Japanese name: 1. Isohaze; 2. Hanahaze; 3. Mimizuhaze; 4. Shirokurahaze; 5. Sejirohaze; 6. Himohaze; 7. Misaki-sujihaze; 8. Kumohaze; 9. Himehaze; 10. Gokurakuhaze; 11. Kutsuwahaze; 12. Abehaze; 13. Noborihaze; 14. Ashishirohaze; 15. Mahaze; 16. Chagara; 17. Kinubari; 18. Ryuguhaze; 19. Agohaze; 20. Dorome; 21. Sabihaze; 22. Chichibu; 23. Shimahaze.

They are obtained from the following localities; Uchiura Bay, Tayu Point and Tateyama Bay (Chiba Pref.); Hirasaku River (Kanagawa Pref.); Haneda, Izumi-tamagawa, and Ara River (Tokyo Metrop.), in 1934, 1935 and 1947. The total length, locality and date of each specimen are all given in the Table 1.

6. TOMIYAMA, I., 1936. Gobiidae of Japan. Jap. Journ. Zool., 7(1), 37-112, figs. 44.

The scales dealt with herein are mostly taken off from the left side of the specimens, and separately from the top or nuchal region of the head, the region above pectoral of the trunk, and the central area of the tail, unless otherwise stated.

The length of cycloid scale is represented by the axial length between the anterior and posterior margins. In the ctenoid scale, however, the posterior border being not so distinct, its length measures from the anterior rim to the inside base line of spine row instead of measuring to the posterior margin of the scale. The width measures at the widest part of the scale.

When the ctenoid scales mingle with the cycloid ones on a bodily part, usually on trunk, the problem will be the intermixture rate of them on the part in question. The rate is referred as the frequency rate of ctenoid scale, or the percentage of ctenoid scale in the test.

The specimens are shown their length with caudal fin or by the total length, or, if not, by the body length under noted so particularly.

Terminology of Scale

The elements of the scale have been termed in various way by many investigators, and the present writer adopts hereupon such terminology as shown in Fig. 1, and some provisions will be offered as follows:

The theoretical focus (*f*) must indicate the original point on which was at first conformed the scale in question. Not being able to recognize the point, however, the present writer practically defines it the center of so-called focal region. This is a round area surrounded by the innermost circulus, being to contain the focus in centre. Circuli are the fine, circular or semi-circular lines lying thickly and concentrically around the focus. Also such two terms as growth zone and resting zone are used, where the circuli are distributed more sparsely (*g-g*) or more densely (*r-r*) respectively.

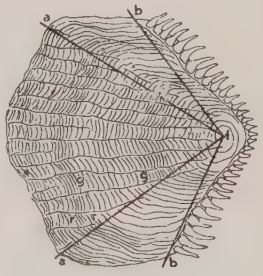


Fig. 1. General appearance of the scale, especially of the ctenoid style. *f*, focus; *g-g*, growth zone; *r-r*, resting zone. Margins: *a-a*, anterior margin; *a-b*, lateral margin; *b-b*, posterior margin. Regions: *a-f-a*, anterior region; *a-f-b*, lateral region; *b-f-b* (the rest of the parts given above), posterior region.

General Feature of Scale

So far as the present investigation goes, the scales are recognized to exist in all but two gobies of *L. guttatns guttatus*, and *E. gilli*. The scale is rather variable in feature, of which we find generally normal and abmormal types. Those two come out in various way, by body parts as well as by species. They are classified after all under the major types of two, viz., cycloid and ctenoid scales (Table 2). It is interesting that both these types appear on the same

species in general, and that one of them scarcely have the whole scaly region to itself (Fig. 2); so the cycloid scales are only seen in such four species as *V. hanae*, *A. lacticella lacticella*, *A. lacticella cosmurus*, and *C. gulosus*; the ctenoid scale exclusively in these three: *E. abax abax*, *Z. farcimen*, and *R. gymnauchen*; and both of them in fourteen species of the rest: *B. fuscus*, *R.*

Table 2. Type distribution in three parts of body. Cy, cycloid scale; Ct, ctenoid scale. Three numerals in parentheses indicate the frequency of the scale variation; normal and abnormal scales, and their intermediate, which is put between them. Sign and numerals in brackets of the column 'trunk,' those of the region covered by pectoral. When the count is not available, only a sign is given, without numerals.

Species	T.L.	Head	Trunk	Tail
A. lacticella cosmurus	42.4	———	———	Cy 18 (14-0-4)
V. hanae	96.3	———	Cy	Cy
A. lacticella lacticella	44.6	———	Cy 13 (10-0-3)	Cy 9 (7-0-2)
C. gulosus	98.1	Cy	Cy	Cy
P. elapoides elapoides	67.2	Cy	Cy {Cy 12 (12-0-0)} {Ct 16 (14-1-1)}	Cy
	79.0	Cy	Cy {Ct 10 (6-2-2)}	Cy
P. zacalles	138.8	Cy	Cy	Cy 63 (0-2-61) Ct 32 (5-3-24)
C. dolichogunathus	64.5	Cy	Cy 35 (3-9-23) Ct 4 (4-0-0)	Cy 18 (2-5-11) Ct 7 (6-0-1)
T. obscurus obscurus	88.3	Cy 25 (19-0-6)	Cy 23 (20-0-3) Ct 18 (16-0-2)	Ct 91 (15-74-2)
M. abei	32.2	Cy 22 (22-0-0)	Cy 9 (9-0-0) Ct 8 (8-0-0)	Ct 32 (22-7-3)
P. elapoides elapoides	42.8	Cy 17 (17-0-0)	Cy 8 (7-1-0) Ct 10 (9-1-0)	Ct 35 (31-1-3)
T. trigonocephalus	61.5	Cy	Cy 6 (5-0-1) Ct 15 (7-0-8)	Ct 81 (78-1-2)
A. lactipes	44.0	Cy	Ct 26 (13-0-13)	Ct 40 (18-0-22)
B. fuscus	48.2	Cy 34 (22-6-6)	Cy 7 (2-3-2) Ct 18 (3-8-7)	Ct 18 (6-4-8)
E. abax abax	41.7	———	Ct 14 (11-3-0)	Ct 20 (16-3-1)
	44.0	———	Ct 15 (13-2-0)	Ct 21 (16-1-4)
Z. farcimen	32.6	———	Ct 11 (7-3-1)	Ct 36 (10-21-5)
R. gymnauchen	76.2	———	Ct 13 (11-0-2)	Ct 25 (19-3-3)
R. giurinus	62.0	Cy 2 (1-0-1) Ct 7 (7-0-0)	Ct 12 (6-0-6)	Ct 10 (9-0-1)
	79.3	Cy	Ct 27 (0-0-27)	Ct
A. ornatus campbelli	94.7	Cy 35 (7-6-22) Ct 7 (7-0-0)	Ct 22 (16-0-6)	Ct 23 (10-11-2)
A. flavimanus	122.9	Cy 10 (4-0-6) Ct 9 (4-0-5)	Ct 24 (13-4-7)	Ct 40 (14-9-17)
O. acutipennis	58.7	Cy 3 (2-0-1) Ct 12 (12-0-0)	Ct 13 (11-2-0)	Ct 32 (27-1-4)
S. genetonema	90.5	Ct 24 (10-0-14)	Ct 22 (15-0-7)	Ct 55 (36-0-19)

giurinus, *A. ornatus campbelli*, *M. abei*, *O. acutipennis*, *A. lactipes*, *A. flavimanus*, *P. elapoides elapoides*, *P. elapoides zonoleucus*, *P. zacalles*, *C. dolichogunathus*, *S. genetonema*, *T. obscurus obscurus*, and *T. trigonocephalus*. In the last case, the cycloid scales occur mostly on the head or the anterior part of the body, and the ctenoid ones on the posterior part of the body. On the area where the two scale zones abut each other, the scales of these two types are seen mixed; on the sides of the head in *S. genetonema*, on the occiput in *A. ornatus campbelli*,

R. giurinus, *O. acutipennis* and *A. flavimanus*, and on the trunk in *M. abei*, *P. elapoides zonoleucus*, *T. obscurus obscurus*, and *T. trigonocephalus*. On the trunk and tail of *C. dolichogunathus* are seen both types of scale, while on the occiput the cycloid scale only. It is especially observed that in *P. elapoides elapoides* and *P. zacalles*, as mentioned by JORDAN and SNYDER (1901)[7], on the region covered by pectoral the ctenoid scales mingle with the cycloid scales, which are exclusive at least on the rest of the abdominal part.* In *A. lactipes*, the cycloid scale region of the occiput extends to almost anterior part of the dorsal insertion.

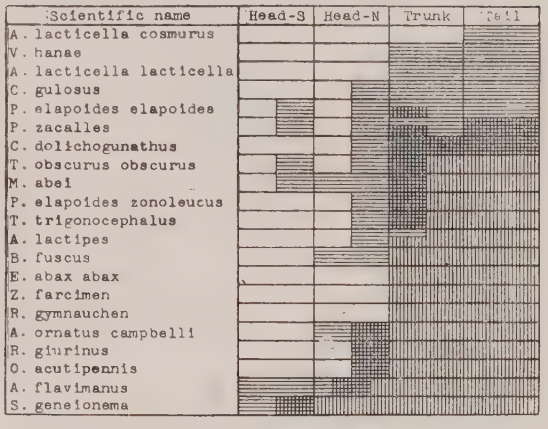


Fig. 2. Distribution diagram of scale types. Chequers, cycloid and ctenoid simultaneously; lateral stripes, cycloid scales; virtical stripes, ctenoid scales. Head-N, nuchal (anterior half) and occipital (posterior half) regions; Head-S, cheek (anterior half) and opercular (posterior half) regions.

Configuration of scale: Scale configuration is so much variable with the spot on which the scale lies, even in the same species. As for the normal scales, there are four forms in general, viz., circular, pentagonal, hexagonal, and broad-bean shaped ones. General speaking the round scales are smaller, and the hexagonal ones greater. It is interesting that the hexagonal scales appear mostly on the trunk and tail, and the rest on the head generally (Table 3): round shape is seen wholly on the body in *V. hanae*, *A. lacticella lacticella*, *A. lacticella cosmurus* and *C. gulosus*, on the head in *B. fuscus*, *R. giurinus*, *A. ornatus campbelli*, *M. abei*, *A. lactipes*, *A. flavimanus*, *P. elapoides zonoleucus*, *T. obscurus obscurus* and *T. trigonocephalus*, and on the tail in *P. zacalles* and *C. dolichogunathus*. The broad-bean shape, which is rather peculiar from among them, on the occipital in *A. flavimanus*.

Scale margin: The anterior margin always more or less waves, when it crosses with the radii, by convexing between two adjoining radii. Here is the anterior angle, in such scales as pentagonal one which is almost rectilineal at the posterior margin, and hexagonal one which has besides the posterior angle. The lateral margins are slightly convex or almost straight. They show a tendency to expand forwards or afterwards in the former case, or to parallel

7. JORDAN, D. S. and SNYDER, J. O., 1901. A review of the gobioid fishes of Japan, with descriptions of twenty-one new species. Proc. U. S. Nat. Mus., 24 (1244), 33-132, figs. 33.

* The present observation of *P. zacalles* can not go so enough, as the specimen is malpreserved.

with the median line of the scale in the latter case. When the scale has the lateral radii, the lateral margin waves as well as on the anterior margin. The posterior margin is round in the circular scale, or pointed at the posterior angle in the hexagonal one. As for the abnormal scale, however, the angle can not be formed even in such scale as ought to have it, and the posterior margin is round as a rule. (Pl. XIV, K–M.)

Table 3. Form distribution in two major scale types, by region and by species. H, nape; Ho, occipital region; Ta, tail; Tp, region covered by pectoral; Tr, trunk. The species with no sign belong to a unique form wholly on the body.

	Circular	Pentagonal	Hexagonal	Broad-bean shaped
Cycloid	*V. hanae* *A. lact. cosmurus* *A. lact. lacticella* *C. gulosus* *P. elap. elapoides*, H *B. fuscus*, H *R. giurinus*, H *A. orn. campbelli*, H *M. abei*, H *A. lactipes*, H *A. flavimanus*, H *P. elap. zonoleucus*, H *P. zacalles*, H *T. trigonocephalus*, H *C. dolichogunathus*, H, Tr *T. obs. obscurus*, H, Tr		*A. orn. campbelli*, T *M. abei*, Tr *P. elap. zonoleucus*, Tr *T. obs. obscurus*, Tr *T. trigonocephalus*, Tr *P. zacalles*, Tr, Ta *C. dolichogunathus*, Ta	
Ctenoid	*A. orn. campbelli*, H *C. dolichogunathus*, Tr	*A. flavimanus*, H *P. elap. elapoides*, Tp *A. orn. campbelli*, Tr, Ta	*E. abax abax* *Z. farcimen* *R. gymnauchen* *R. giurinus* *O. acutipennis* *S. geneionema* *B. fuscus*, Tr, Ta *M. abei*, Tr, Ta *A. lactipes*, Tr, Ta *A. flavimanus*, Tr, Ta *P. elap. zonoleucus*, Tr, Ta *T. obs. obscurus*, Tr, Ta *T. trigonocephalus*, Tr, Ta *P. zacalles*, Ta *C. dolichogunathus*, Ta	*A. flavimanus*, Ho

Spine: The spines are generally fine, and canine-like, and distributed in a row, wholly or partly on the posterior margin, except those on occipital scales of *A. flavimanus*, which grow in rows. They are sometimes uniform in size on their row, or sometimes diminish with receding from the posterior angle to the postero-lateral corners. The latter case is seen only in such scales having the posterior angle and many spines as those of the hinder parts of body of *E. abax abax*, *B. fuscus*, *R. gymnauchen*, *R. giurinus*, *A. ornatus campbelli*, *M. abei*, *O. acutipennis*, *A. lactipes*, *A. flavimanus*, *S. geneionema*, *T. obscurus obscurus*, and *T. trigonocephalus*. While even in this case, the spines of abnormal scales are larger than as usual, tend to be uniform size, and appear grow in

rows because of having some, not so many, minute spines on foot of them. (Pl. XIV, K–M.)

The number of spine is very variable. It seems to be very important as a specific character. As for caudal scales, the number is very small, below 15, first of all in *P. zacalles* and *C. dolichogunathus*, as well as in *Z. farcimen* and *P. elapoides zonoleucus*; and above 30 in the other species: 30–32 in *M. abei*, *A. lactipes*, *S. geneionema*, and *T. trigonocephalus*, 44 in *O. acutipennis*, 50–60 in *E. abax abax*, *B. fuscus*, *R. gymnauchen*, *R. giurinus*, *A. flavimanus*, and *T. obscurus obscurus*, and about 80 in *A. ornatus campbelli* (Table 4).

Table 4. Number of radius (R) and spine (S) of caudal scales.

Items	Species	R	S
Cycloid	*A. lacticella cosmurus*	10	
	A. lacticella lacticella	10–13	
	V. hanae	11	
	P. elapoides elapoides	11	
	C. gulosus	19	
Ctenoid	*P. elapoides zonoleucus*	10	9
	S. geneionema	9–10	32
	Z. farcimen	11	11
	P. zacalles	13	5
	A. ornatus campbelli	15	80
	A. lactipes	17	32
	T. trigonocephalus	18	30
	M. abei	19	31
	A. flavimanus	19	51
	E. abax abax	19	54
	B. fuscus	20	51
	C. dolichogunathus	21	6
	R. gymnauchen	22	57
	O. acutipennis	23	44
	T. obscurus obscurus	24	51
	R. giurinus	25	60

Focus: The focus is situated close to the posterior margin, or apart from the margin to near the scale center. All the ctenoid scales and a small number of the cycloid ones are of the former case, as seen wholly on the bodily parts in *A. lacticella lacticella*, *C. gulosus*, *E. abax abax*, *Z. farcimen*, *B. fuscus*, *R. giurinus*, *A. ornatus campbelli*, *O. acutipennis*, *A. lactipes*, *P. zacalles*, *T. obscurus obscurus*, *S. geneionema*, and *A. flavimanus**, on the trunk and tail in *M. abei*, *C. dolichogunathus*, *P. elapoides zonoleucus**, and *T. trigonocephalus*, on the region covered by the pectoral and the tail in *P. elapoides elapoides*, and on the tail in *V. hanae*. The cycloid scales on the head and a few of those on the trunk and tail are of the latter case; on the head in *M. abei*, *A. flavimanus* (especially on occiput), *C. dolichogunathus* and *T. obscurus obscurus*, on the head and trunk in *P. elapoides elapoides*, *P. elapoides zonoleucus*, and *T. trigonocephalus*, on the trunk in *V. hanae*, and on the tail in *A. lacticella cosmurus*.

* We mention here of the ctenoid scale only in these species.

Circulus, growth zone and resting zone: The circuli seem to consist of series of uplifting lines. General speaking their distribution is thicker on the anterior region than on the other regions, where they reduce in number.

The growth and resting zones are at any rate discerned in large number of species, especially in *R. giurinus*, *A. ornatus campbelli*, *A. flavimanus*, *C. dolichogunathus*, *S. geneionema*, and *T. obscurus obscurus*. In *B. fuscus*, one of the circulus interval is about two times broader than the others and makes an exceptional broad band on the scale (Pl. XII, G). It should not be decided easily if it indicates the so-called spawning mark. No resting zone nor growth zone is found in *V. hanae*, *A. lacticella lacticella*, *A. lacticella cosmurus*, *P. elapoides elapoides*, and *P. elapoides zonoleucus*, though dealt with the scales from rather grown up individuals, except those of the last species.

Radius: The radii are found on the anterior and lateral regions running radially from the focus outwards, and, as far as the present study goes, never diverge but simple. They are distributed on the anterior region in general excepting the scales of the whole body in *V. hanae*, *A. lacticella lacticella*, *A. lacticella cosmurus*, *C. dolichogunathus*, and *C. gulosus*, of the head in *R. giurinus*, and the cycloid ones of the head and trunk of *T. obscurus obscurus*. The radii extend to the lateral region on these species.

As for the caudal scale, the number of radius varies about 10 to 25 on the whole; the minimum is 9 in *P. elapoides zonoleucus*, the maximum 25 in *R. giurinus*. In the cycloid scales, the number is always rather small, about 10, with the exception of *C. gulosus*, in which it is so many as 19; in the ctenoid ones it counts rather many, exceeding to 20 in *B. fuscus*, *R. gymnauchen*, *R. giurinus*, *O. acutipennis*, *C. dolichogunathus*, and *T. obscurus obscurus* (Table 4). The interrelationship between the number of spine and radius is not found to exist. In any way, the number of radius seems to have less importance as a taxonomic character of the scale than the spine number.

Scale Types and Their Phylogenetic Relationships

After investigating comparatively the scales on each body part, the writer can not help conclusing that the caudal scale is the representative of those of each species. As for the caudal ones, their configurations are classifiable to four types, A to D; the former two are referred to the cycloid scale, and the rest to the ctenoid one. By the type-A are embraced such circular or elliptic scales as seen in *V. hanae*, *A. lacticella lacticella*, *A. lacticella cosmurus*, and *C. gulosus*; by the type-B is represented the hexagonal one of the single species, *P. elapoides elapoides*. The type-C and D are seen in the hexagonal and pentagonal ones having a spine row on or inside the posterior margin. Of these latter, the type-C embraces the scales which have hardly developed spines and belong to two species: *P. zacalles* and *C. dolichogunathus*. The D-type scale, having spines remarkably developed and large in number, represents the rest of all, 10 species and 4 formae, which are 66.6% of the scaly fishes here examined. After all the large number of the gobiid fishes seem to be endowed with the D-type scale.

The phylogenetic relationships of the scale are considered on the basis of such characters as their shape, their spines on edge, etc. In process of the development of the scale, the new-born scale is cycloid and almost circular, even in case of the ctenoid one as well as of the cycloid one. It seems to be easily affirmed in view of this fact that the primitive scale is a cycloid one, especially that of circular. We have seen after Ishikawa and Nakamura[1] that the spines of *G. giuris brunneus* is small in number in its earlier stage of development and that they increase in number with their growth, which may exactly indicate the secondary formation of the spine. Upon the viewpoint of this the phylogenetic relationships of the scale-types are induced as follows:

Of these four scale-types, the type-A is the most primitive one, the type-B is a rather differentiated form of the type-A, and the type-D is that being in process of the most specialization, so the type-C is an intermediate form of the types-B and D.

Key to The 21 Gobies Based on Features of Caudal Scale

I. Cycloid scale exclusive.
- a^1 More or less circular, or elliptical (Type-A).
- b^1 Circular, and the rate of width and length, 0.91-1.10.
- c^1 Outermost radii cross rightly to the ordinate of scale; radius number, 11. .....*Vireosa hanae* 1.
- c^2 Outermost radii ran obliquely in the lateral region; radius number, 10. *Astrabe lacticella cosmurus* 2.
- b^2 Elliptical, and the rate of width and length, 0.68-0.78.
- d^1 Radius number, 10-13. .....*Astrabe lacticella lacticella* 3.
- d^2 Radius number, 20. .....*Chasmichthys gulosus* 4.
- a^2 Hexagonal (Type-B).
  Rate of width and length, 0.69-0.80; radius number, 11. ..... *Pterogobius elapoides elapoides* 5.

II. Ctenoid scales exclusive, or mingle more or less with cycloid ones.
- a^1 Spines microscopical, growing near to the focal region rather than posterior margin as usual; small in number, at most to be several (Type-C).
- b^1 Scales elongated hexagonal. .....*Pterogobius zacalles* 6.
- b^2 Scales almost regularly hexagonal with dull corners. ..... *Chasmichthys dolichogunathus* 7.
- a^2 Spines eminent, growing on posterior margin; ten or more in number (Type-E).
- c^1 Spines rather small in number; not more than 15.
- d^1 Scales elongated hexagonal. .....*Pterogobius elapoides zonoleucus* 8.
- d^2 Scales broader than length; hexagonal with dull corners..... *Zonogobius farcimen* 9.
- c^2 Spines numerous, at least 30.
- e^1 Proportion of width and length of scale, 0.81; number of radius, 9-10; number of spine, 32. .....*Sagamia geneionema* 10.
- e^2 Proportion, 0.84-0.99 (average 0.91); radii, 15; spines, 80. ..... *Acentrogobius ornatus campbelli* 11.
- e^3 Proportion, 0.84-1.02 (average 0.95); radii, 25; spines, 60. ..... *Rhinogobius giurinus* 12.
- e^4 Proportion, 0.85-1.20 (average 0.97); radii, 20; spines, 51. ..... *Bathygobius fuscus* 13.
- e^5 Proportion, 1.03; radii, 24; spines, 51.....*Tridentiger obscurus obscurus* 14.

e[6] Proportion, 1.05; radii, 17; spines, 32. .....*Aboma lactipes* 15.
e[7] Proportion, 0.99-1.21 (average 1.10); radii, 22; spines, 57. .....
Rhinogobius gymnauchen 16.
e[8] Proportion, 1.13; radii, 19; spines, 50. .....*Acanthogobius flavimanus* 17.
e[9] Proportion, 1.15-1.21; radii, 18-19; spines, 30-31 .....
Mugilogobius abei 18. *Tridentiger trigonocephalus* 19.
e[10] Proportion, 1.23; radii, 23; spines, 44. .....*Oligolepis acutipennis* 20.
e[11] Proportion, 1.25; radii, 19; spines, 54. .....*Eviota abax abax* 21.

Descriptive Part

1. *Vireosa hanae* (JORDAN et SNYDER)
(Pl. X, A; Pl. XII, C.)

Scale minute, oval, seems to be completely embeded. Caudal scales larger and rather broader than abdominal ones. Size 0.20–0.29×0.21–0.29 (0.23×0.26) mm* on trunk, 0.28–0.33×0.31–0.35 (0.31×0.33) mm on tail of the specimen of 96.3 mm long. Circuli laid down rather sparsely on all surface including that of posterior region. Radii well developed on post-lateral region, but rather smal in number, and tend to increase in number from trunk to tail, 7 on the former, 11 on the latter.

So far as the present investigation goes, the scale of this species is well defined from the other scales by the post-lateral radii.

Describing this species, JORDAN and SNYDER[7] (p. 39) write that "head naked," and that "body with minute, cycloid, partly embeded scales, which are smaller anteriorly, and larger posteriorly," and TOMIYAMA[6] (p. 38) also mentions "head naked, body scaly." These descriptions agree with the results taken by the present investigation.

2. *Astrabe lacticella cosmurus* (JORDAN et SNYDER)
(Pl. X, B.)

No scale seen on head and trunk, but sometimes caudal scaly region extends forward to the latter. On the caudal scaly region we prove some cycloid scales; some of them exposed at least on a part. Size 0.29–0.42×0.31–0.36 (0.36×0.33) mm in the specimen of 42.4 mm long. Circuli arranged on nearly all part of scale, but disappear on most part of the posterior region. Radii 10 in number on caudal scales of the specimen given above.

Of the caudal scaly region, TOMIYAMA[6] (p. 53), dealing with some specimens of 15–44 mm long, describes that it extends "anteriorly in a single series to below spinous dorsal origin at most," while it is not found in any specimen here examined (about 40 mm long) to extend forward beyond the begining of the second dorsal.

* The size is shown hereupon by two numerals (or ranges) connected with a cross (×) each other, which represent length and width respectively. The ranges are followed by their averages, in brackets, which are arranged similarly.

3. *Astrabe lacticella lacticella* (Jordan et Snyder)
(Pl. X, C; Pl. XII, D, E.)

Scale rather elliptic, or oval. Caudel scales larger, especially in length, than abdominal ones. Scales measure 0.33–0.39×0.35–0.47 (0.35×0.41) mm on trunk, 0.35–0.48×0.49–0.64 (0.42×0.57) mm on tail of the specimen of 44.6 mm long. Circuli very small in number on posterior region, vanish partly along posterior margin of caudal scales. Radii of the same individual, 10 on trunk and 10–13 on tail in number.

Of the scaly area Jordan and Snyder[7] (p. 120), basing on a specimen of ca. 34.8 mm long (calculated by the present writer from Fig. 26; op. cit., p. 120), describe that "head and anterior parts of naked" and that the scales on posterior parts "grow forward on a V-shaped area almost to base of pectoral." Tomiyama[6] (p. 53), agreeing with them after his observation based on eight specimens of 25–55 mm in body length, describes that "body scaly behind pectoral base." The present writer also consents to them after careful investigation basing on the above specimen and the other one of 49 mm long, and moreover proves a spot bearing a few of the same oval scales as caudal ones above pectoral.

It may be noticed herewith that the present writer has experienced in this study that the body scales of this goby are more easily visible in larger specimen (49.0 mm long) than in smaller one (44.6 mm long). Of this problem, Jordan and Snyder[7] describe that scales are embeded. The specimens dealt with by them are smaller than those examined by the present investigator. Jadging from the matter given above, it may be concluded that the goby has embeded acales in younger stage and afterwards the scales become visible with advance of age.

4. *Chasmichthys gulosus* (Guichenot)
(Pl. X, D; Pl. XIII, K, L; Pl. XVI, K.)

Scale minute; more or less embeded, especially on head; of oval, sometimes narrowed anteriorly; generally narrower on anterior part of body, broader on posterior part. Ratio of width to length, 0.70 on head, 0.62–0.73 (average 0.66) on trunk, 0.76 on tail. Size in the specimen of 98.1 mm long, 0.51×0.72 mm on head, 0.52–0.95×0.83–1.29 (average 0.68×1.01) mm on trunk, 0.87×1.14 on tail. Circuli hardly seen on posterior region. Radii very numerous, 26 and 19 on trunk and tail respectively.

It is worthy of mentioning that the possesion of such many radii as written above is a property distinguishing this goby from other ones with cycloid scale.

Jordan and Synder[7] (p. 87) set forth that head naked, after their specimen of 100 mm long, while the writer get some scales on head of an individual of nearly the same lengths as theirs (of 98.1 mm long).

5. *Pterogobius elapoides elapoides* (Günther)
(Pl. X, E; Pl. XIII, I, J; Pl. XVI, F.)

Scale minute, but visible rather easily with the naked eye except those on head, which are embeded and hardly made out; exclusively cycloid except

for those of the region covered by pectoral which are cycloid and ctenoid. Cephalic scale oval. Abdominal and caudal ones both of prolonged hexagon. Of these two, the former has convex sides and duli angles, the latter having more straight sides and more pointed angles. Size 0.41–0.55×0.57–0.66 (0.46×0.60) mm on head, 0.68–0.74×0.75–0.89 (0.70×0.83) mm on the region above pectoral, and 0.75–1.00×1.08–1.27 (0.90×1.20) mm on tail in the 67.2 mm long specimen; in the other specimen of 79.0 mm long, 0.49–0.56×0.59–0.65 (0.52×0.63) mm, 0.86–1.02×1.04–1.18 (0.94×1.11) mm and 0.98–1.05×1.24–1.33 (1.00×1.29) mm on each part of body respectively. Scales under pectoral measure, cycloid and ctenoid ones alike, 0.86–1.06×1.98–1.07 (0.95×1.02) mm in size in the specimen of 67.2 mm long, and 1.17–1.29×1.07–1.17 (1.23×1.12) mm in the other one of 79.0 mm long, which happens to bear ctenoid scales only. These measurements apparently show that the scales of the region in question are broader than the other ones, and intermediate in length between those of trunk and tail.

Circuli more sparse in distribution on posterior region than on the others of nuchal and abdominal scales, vanish on the marginal part of the region of caudal ones. Radii 6, 7 and 11 in number on nuchal, abdominal and caudal scales respectively, and 11 on scale under pectoral. The rate of ctenoid scale of the region covered by pectoral is very variable on the two specimens dealt with, 57.1% in the smaller one, and 100% on the other.

On the above specimens, ctenoid scales grow nearly anywhere under depressed pectoral. On the smaller specimen, 67.2 mm long, none of ctenoid scales is found to exist below the tip of the fin in question. JORDAN and SNYDER[7] (p. 91), however, mention that "a few ctenoid scales below tip of depressed pectoral," only.

6. *Pterogobius zacalles* (JORDAN et SNYDER)
(Pl. X, F; Pl. XIV, A, B; Pl. XVI, H, I.)

Scale minute but rather easily recognizable. Occipital scales ovoid, rather compressed posteriorly. Abdominal ones show a intermediate form between elliptical and hexagonal ones, having rather dull corners except for anterior one. Caudal scales prolonged hexagonal, with rather dull angles. Size larger on tail than on trunk, whereas the proportion of length to width is nearly the same on both two bodily parts; 0.55×0.66 mm on head, 1.00×1.61 mm on trunk, 1.29×2.02 mm on tail, of the specimen of 138.8 mm long.

Circuli set rather thickly in distribution on nuchal scales, sparsely on both abdominal and caudal scales, especially in their posterior region. Radii varies in number with bodily parts, 7 on head, 11 on trunk except for the region of pectoral, where the number is 19, and 13 on tail.

Spines 5 and 7 in number on caudal scale and that of the region of pectoral respectively, minutely tuberculous on normal caudal scale, instead of being canine-like as seen on abnormal scales under pectoral and on tail in the above specimen.

Although another example will be shown in *C. dolichogunathus*, it appears to be an exceptional character that the spines assum a tuberculous form. But

there is a doubt as given in the following lines. The cteni of the normal caudal scales are tuberculous. Whilst those of abnormal ones are sharply pointed. So that such exceptional form of the cteni of the normal scales may be made up by break of their tip. Notable enough, angles of cudal scales are found blunter than those of any other species. On the region covered by the pectoral, some abnormal scales only happen to be found. But it is assumed that the normal scales are of nearly the same in form as those of the same region of *elapoides*, after the shape of abnormal scales dealt with, on which the spines are seen set on a line.

Jordan and Snyder[7] (p. 93) recognized some ctenoid scales on the particular area covered by the pectoral only, but, as mentioned above, the caudal region bears them too. The cteni of caudal ones, however, are so minute that the above authors mistake these scales for cycloid ones.

7. *Chasmichthys dolichogunathus* (Hilgendorf)
(Pl. X, G; Pl. XIV, C; Pl. XVI, G.)

Scale minute but easily visible; round on head and trunk, hexagonal on tail. Nuchal scales nearly circular, abdominal ones with weakly developed anterior and antero-lateral angles. Caudal ones with rather well developed anterior three corners, rather dull posterior ones, especially in hindermost one.

Size takes minimum on head, and maximum on trunk; 0.52×0.52 mm on head, 1.04×0.95 mm on trunk, and 0.82×0.87 mm on tail, of the specimen of 64.5 mm long.

Circuli rather thick on all but posterior region, where hardly distributed. Resting zone rather clear on abdominal and caudal scales. Radii 12 in number on head, 24 on trunk and 21 on tail. Spine minute, inserted nearer to focus than to posterior margin as in the case of *P. zacalles*, 5–6 in number on both trunk and tail. Frequency rate of ctenoid scale larger on tail than on trunk; 10% in count of 39 abdominal scales, and 28% of 25 caudal ones, while 0% on head. Finally it may be added that 9% are found abnormal on scales of abdominal and caudal parts. These two facts: spine is minute and set nearer to the focus than to the posterior rim of the scale, are characters for the present species as well as *P. zacalles*.

With regard to the scale of this goby, there are none who did its description but Jordan and Snyder[7] (p. 84–85) and Tomiyama[6], since Hilgendorf. Jordan and Snyder say that "head naked," and that "scales on body small and weak, cycloid." But as stated already, small cycloid scales on occiput as shown in Fig. G, Pl. XVI, and ctenoid ones with very minute cteni on both trunk and tail were found to exist.

Tomiyama regards this goby as a form of *C. dolichogunathus*, with the other form of *gulosus*. In fact, they resemble each other in general appearance, but the present goby is clearly distinguishable from *C. dolichoguuathus gulosus* by hexagonal shape of scale and presence of ctenoid scales. Although it remains to study in future, the writer dares to bring forward to raise again each of them to the status of species.

8. *Pterogobius elapoides zonoleucus* (Jordan et Snyder)
(Pl. X, H; Pl. XIII, G, H; Pl. XVI, E.)

All specimens examined hereupon are samely about 40 mm long, although this fish seems to grow up to 70–90 mm in lehgth, according to Jordan and Snyder[7], and Tomiyama[6].

Scale minute, but visible rather easily on trunk and tail, though difficult to be found out on head. Cycloid and ctenoid scales do both grow on this goby as well as in large number of the gobies dealt with; on head and tail exclusively found cycloid and ctenoid ones respectively, while on trunk shown both of them. Rate of ctenoid scale 55.5% in this case. Shape varies with bodily parts, elliptic on head, hexagonal on the other parts of body. As for abdominal scales, cycloid scales as long as wide, having blunter corners than those of ctenoid ones; ctenoid ones rather broader as in those on the tail of *Z. farcimen*. In caudal scales anterior three angles more or less sharper than those of abdominal ones; and their width increases much more than their length do towards posterior end of body.

Size smallest on head, largest on tail; in the specimen of 42.8 mm long, 0.31–0.37×0.39–0.49 (0.33×0.45) mm on head, 0.52–0.57×0.52–0.55 (0.54×0.53) mm and 0.66–0.73×0.62–0.67 (0.70×0.63) mm on the cycloid and ctenoid scales of trunk respectively, and 0.65–0.74×0.76–0.84 (0.67×0.79) mm on tail.

Circuli normal in distribution on all regions except for posterior one, on which rather sparse. Radii 5, 7, 9, and 10 in number on head, trunk on both type of cycloid and ctenoid types, and tail respectively.

The circuli are distributed more sparsely on the posterior region than on all other regions. This tendency is found stronger on the cycloid scales.

Jordan and Snyder[7] (p. 95) refer to this subject and say that "body eveywhere covered with small, finery ctenoid scales." So far as the present investigation goes, however, as noted above, both types of scale are detected mixed on trunk at least on its anterior part.

9. *Zonoleucus farcimen* (Jordan et Evermann)
(Pl. X, I; Pl. XII, F.)

Scale rather large, exfoliate easily, belonging to the type–D except for some abnormal ones, a little wider than length, rather round than hexagonal, because of degenerated angles, showing a intermediate form of widely elliptical and hexagonal scales. Size 0.57–0.69×0.72–0.92 (0.62×0.79) mm on tail. Circuli set rather thickly, as well as those of *C. dolichogunathus* and *P. zacalles*. Radii 16 in number on caudal scale of 37.1 mm long specimen, and 11 of 32.6 mm long one. Spines 8–11 in number.

10. *Sagamia geneionema* (Hilgendorf)
(Pl. X, J; Pl. XIV, D; Pl. XVI, L–O.)

Scale rather small, but visible with the naked eye, except for embeded scales of head side, both cycloid and ctenoid on head, wholly ctenoid on trunk

and tail. In head part, on cheek grow cycloid scales solely; on upper margin of operculum both cycloid and ctenoid ones, about 30% in frequency rate of ctenoid scale. The rest of the body covered exclusively with ctenoid scales.

Shape hexagonal on all regions but head side, showing some differences by bodily parts in proportion and sharpness of angles. On side part of head, it is irregular showing hexagon, oval or of their intermediations. Hexagonal ones have length equal to or more than width, and blunt corners, anterior angle of which is hardly developed. Abdominal scales as long as width. Caudal scales longer than width. Abdominal and caudal scales resemble in general feature those of *P. elapoides zonoleucus*, but differ from the latter ones in possession of sharper posterior angle and much larger number of spines. Size of scale largest on trunk, measures 1.45×1.39, 1.70×1.70, 1.43×1.75 mm on nuchal region, trunk and tail respectively in the specimen of 90.5 mm long.

Circuli laid thickly as well as in *Z. farcimen*. Resting zones are seen rather clearly, especially on cephalic scales. Radii unique on all bodily parts, 10–12 in number. Spines rather smaller in number on nuchal region than on both trunk and tail, as bearing 25 on the former one, 35 and 25 respectively on the latter two.

The specimen dealt with of 90.5 mm long bears some scales on nuchal region and cheek, as mentioned above. Jordan and Snyder[7] (p. 110), however, say that "head naked, except occiput and upper edge of opercle," based on smaller specimens of 37–54 mm long. These difference occur probably from the distinction of age, after taking into considerations that the former workers treat much smaller specimens than the present writer's.

11. *Acentrogobius ornatus campbelli* (Jordan et Snyder)
(Pl. X, K; Pl. XIII, A–D; Pl. XV, E–J.)

Scale large, ctenoid only on all but nuchal region, which bears both types of scales. Frequency rate of ctenoid scale of the region, 16.6% in count of 42 scales. Nuchal scales vary in form, of petal-like shape narrowed in posterior part and of hexagon. Ctenoid scales appear there mostly by the former shape. Abdominal and caudal scales are of pentagon furnished with evenly flat anterior rim and well developed posterior angle. Size, in the fish of 94.7 mm long, 2.37×3.09 mm and 2.40×3.51 mm on cycloid and ctenoid ones of nuchal region respectively, 3.62–4.21 × 3.70–4.37 (3.90 × 4.01) mm on trunk, and 4.00–4.58 × 4.20–5.01 (4.24 × 4.60) mm on trunk; among these, caudal scales more or less larger than abdominal ones.

Circuli found rather thickly on distribution, covering all area of scale, but rougher in number on lateral region. Resting zones present. Radii never distributed on lateral region, not so vary in number with bodily parts bearing the scale, 11 and 9 in number on cycloid and ctenoid scales on head respectively, 12 on trunk, and 15 on tail. Spine minute, counted 26 on nuchal scale, 82 on abdominal one, 80 on caudal one in number. The last number is the maximum through all the gobies dealt with herein.

JORDAN and SNYDER[7] (p. 62) indicate that "those[=scales] on nape and occiput cycloid," but some ctenoid scales found to exist besides cycloid ones on the regions in question as denoted already.

12. *Rhinogobius giurinus* (RUTTER)
(Pl. X, L, M; Pl. XII, I; Pl. XV, B, C.)

Of the specimens dealt with, the 79.3 mm long one bears mostly abnormal scales with circuli confused on focal region, and no normal scale on trunk, and the other one is as usual.

Scale moderate in size, embeded on head, exposed on the rest. Cycloid scales on head broadly circular in shape; ctenoid ones hexagonal with rather sharp posterior angle on whole body. Size, 1.38×1.05 mm and 1.22–1.55 × 1.05–1.14 (1.39×1.09) mm on head, on cycloid and ctenoid scales respectively, 1.78–2.81 × 1.67–2.38 (2.23×1.93) mm on trunk, 2.45–2.88 × 2.35–2.51 (2.64×2.46) mm on tail in the 62.0 mm long specimen; 0.82×0.67 mm on head (cycloid one only), 1.85–2.38 × 2.09–2.38 (2.12×2.21) mm on tail in another specimen of 79.3 mm long.

Circuli arranged rather thickly on anterior region and more or less roughly on lateral regions. Resting zones present; four zones were found on a caudal scale of the specimen of 79.3 mm long. Radii 18 in number on nuchal cycloid scale, and 25 on caudal ctenoid scale. Spines minute, and numerous, about 60 in number on tail. These numbers are also related to the larger individual.

As for the larger specimen, it may be remarkable that one to four transparent disklets, measuring 0.12×0.13 to 0.19×0.27 mm in size, are found lying on outer surface of scales of the trunk and tail. These disklets seem to be homogeneous with scale. On the trace of them after scratched are hardly seen such sculptures as circuli and radii. (Pl. X, L, M.)

13. *Bathygobius fuscus* (RÜPPELL)
(Pl. XI, A, B; Pl. XII, G; Pl. XV, A.)

Scale rather small, cycloid on head, ctenoid on tail, both cycloid and ctenoid and on trunk. Rate of ctenoid scale on trunk varies with individual, 51.7% and 72.0% on two present specimens respectively. Nuchal scales oval, narrowed posteriorly. Abdominal and caudal ones hexagonal in outline, as long as width, somewhat trifoliate on anterior border, more or less sharply pointed on posterior angle. Size augments backwards as indicated already by JORDAN and SNYDER[7] (p. 53) running as follows; 0.72–0.89 × 0.77–0.95 (0.80×0.86) mm on head, 1.56–1.71 × 1.41–1.58 (1.63×1.51) mm on trunk, 2.24–2.82 × 2.24–2.40 (2.59×2.30) mm on tail, in 62.5 mm long specimen.

Circuli set rather sparsely. Resting zone absent, but a broad band running between circulus rows on abdominal and caudal scales (Pl. XII, G). Radii and spines show a tendency to increase in number towards posterior part of body. The former 13, 15 and 20 in number on head, trunk and tail respectively. The latter 46 on trunk, 51 on tail in number.

Remarkably enough, the band found on the scales of the trunk and tail takes place near the margin of those scales in the two species of 48.2 and 56.9 mm long, and it is found lying near the middle between the focus and the anterior edge of the scale in the specimen of 62.5 mm long. On the other hand, the former two specimens are secured in May, and the other one in June, though they were not taken in the same year. Accordingly it seems that the bands in question were achieved during a period from winter to spring.

14. *Tridentiger obscurus obscurus* (Temminck et Schlegel)
(Pl. XI, C; Pl. XIV, E–G; Pl. XVI, P, Q.)

Scale large, but smaller than such as in *A. ornatus campbelli;* cycloid and ctenoid: cycloid on head and ctenoid on tail exclusively, and of both types on trunk. Frequency rate of ctenoid scale on trunk 43.9% in count of 41 scales. Shape varies with bodily parts; circular or oval on head, circular in cycloid scale and hexagonal in ctenoid one of trunk, hexagonal on tail. Size measures in cycloid scales, 0.67×0.92 mm on upper margin of operculum, 1.10×1.08 mm on occiput, 1.84×1.64 mm on trunk; in ctenoid scales, 1.98×1.72 mm and 2.42×2.40 mm on anterior and posterior regions of trunk respectively, and 2.75×2.66 mm on tail of the specimen 88.3 mm long. Circuli set rather sparsely; resting zones rather defind, two zones found on scales of specimen, 88.3 mm long. Radii large in number, 18 on head, 22–24 on trunk and tail respectively. Spines grow partly on the middle of posterior margin, 28 in number, on the forepart of trunk; almost wholly along posterior margin as usual, 51–55 in number on the rest of bodily parts.

On abdominal cycloid scales, smaller type scales are found to exist besides larger type ones, of which the size is given above. The small type ones occur about two times frequently than the other ones.

Also it may be mentioned herein that the present species stands next to *R. giurinus* in largeness of the radius number within the limit of this scrutiny.

Jordan and Snyder[7] (p. 114) describe that "head naked .... Body with large, ctenoid scales ...." It is revealed, however, that the present specimens have some embeded cycloid scales on the occiput and near upper margin of the operculum, and exposed ones on the anterior part of the trunk where both cycloid and ctenoid scales are found mingled in the ratio of about 50%.

15. *Aboma lactipes* (Hilgendorf)
(Pl. XI, D; Pl. XII, M; Pl. XV, M.)

Scale embeded on head, partly on trunk and tail; cycloid on head, ctenoid on the rest; longer than broad, oval on head, hexagonal on trunk and tail, but becomes elongate with sharper anterior and posterior angles on hinder part of body. Size moderate, 0.77×0.54, 1.22×0.84 and 1.44×1.37 mm on head, trunk and tail respectively, in the specimen of 44.0 mm long.

Circuli laid rather rouglhy, but roughly in lateral regions. Resting zone not found. Radii 11, 13 and 17 in number on head, trunk and tail respectively.

Of this species, Jordan and Snyder[7] (p. 68) make descriptions as follows: "head naked," and "body with large, finery ctenoid scales." It has been above mentioned that the present specimen of 44.0 mm long has some cycloid scales on head. This nuchal cycloid scales are arranged backward to the trunk, above pectoral, where the ctenoid scales are to grow after the above authors.

16. *Rhinogobius gymnauchen* (Bleeker)
(Pl. XI, E; Pl. XII, H.)

Scale large, exposed; ctenoid exclusively on trunk and tail, broader than long, with sharper posterior angle on hinder bodily part. Size 2.23–2.75 × 1.85–2.23 (2.49 × 2.05) mm on trunk and 2.51–3.04 × 2.28–2.77 (2.74 × 2.48) mm on tail, in the two specimens of 72.7 and 76.2 mm long.

Circuli set more or less thickly in distribution but rather sparsely on lateral regions. Resting zone not found. Radii larger in number posteriorly, 17 on trunk and 22 on tail. Spines minute, numerous as well as *R. giurinus* or *E. abax abax*, 45 on trunk, 57 on tail.

17. *Acanthogobius flavimanus* (Temminck et Schlegel)
(Pl. XI, F, G; Pl. XIII, E, F; Pl. XV, N, O; Pl. XVI, A–D.)

Scale moderate in size, exposed anywhere but embeded on head sides; cycloid and ctenoid on head, exclusively ctenoid on trunk and tail; variable in shape, especially on head bearing circular, oval, pentagonal and broad-bean like scales, broadly hexagonal on the rest of body. Shape mostly oval in cycloid scales of cheeks and nuchal region, pentagonal or broad-bean shaped in ctenoid ones arranged in a file along lateral rims of nuchal region, upper ridge of operculum, and on occipital region. Frequency rate of ctenoid scale 50% on head. Size smaller on head than on the rest of body, alike on trunk and tail, 0.90 × 0.83 mm on cheek, 1.11 × 1.00 mm on upper part of operculum, 1.25 × 1.56 mm and 1.74 × 1.36 mm on nuchal region (cycloid and ctenoid scales respectively), 3.12 × 2.19 mm on trunk, 2.96 × 2.61 mm on tail of the specimen of 122.9 mm long.

Circuli set on whole surface of scale with weaker distribution on lateral regions than on anterior region as mentioned by Miyazaki[2]. Resting zone vague. Radii 11–12 in number on nuchal region, 14 on cheek, 9 on upper margin of operculum, 16 on trunk, 19 on tail. Spines lined up along posterior margin of scale, except for those of occipital scales of pentagonal and broad-bean shape, which grow in rows; 55 in number on trunk, 51 on tail.

Miyazaki[2] indicates that the focal region is found extraordinary broad in some scales, and that circuli are in disorder around it. Such adnormal scales are also seen in this case in the proportion of 30% on trunk and 42% on tail.

18. *Mugilogobius abei* (Jordan et Snyder)
(Pl. XI, H ; Pl. XII, J, K ; Pl. XV, D.)

Scale minute, but visible with ease with the naked eye, cycloid on upper part of operculum, nuchal region and trunk. Ctenoid on trunk and tail ; 47% in count of 17 scales in frequency rate of ctenoid scale on trunk ; oval in outline on head, hexagonal with obtuse anterior angle on trunk and tail. Caudal scales asymmetrically distorted in shape ; among them scales lying above the median axis of body have their longitudinal axis biassed towards ventral edge, and on the contrary those placed below the median body-axis are provided with their longitudinal one deviated towards dordal border (Pl. XI, H). Size "small anter iorly, growing gradually larger posteriorly," as pointed out already by Jordan and Snyder [7], measures 0.70×0.59 mm on head sides, $0.59–0.74 \times 0.56–0.66$ (0.67×0.62) mm and $0.74–0.92 \times 0.66–0.74$ (0.82×0.70) mm on trunk (cycloid and ctenoid scales respectively), $0.99–1.43 \times 0.90–1.18$ (1.18×1.01) mm on tail in the specimen, 3.22 mm long.

Circuli arranged on whole surface of scale, lying more sparsely on lateral regions than on anterior one with the exception of abdominal scales, on which they vanish posteriorly. Both radii and spines larger in number ; the former 11–12 on trunk, 19 on tail ; the latter 22 and 31 on trunk and tail respectively.

19. *Tridentiger trigonocephalus* (Gill)
(Pl. XI, I ; Pl. XIV, H, I ; Pl. XVI, J.)

Scale moderate in size, cycloid on occiput and trunk, ctenoid on trunk and tail. Abdominal cycloid scales generally smaller than abdominal ctenoid ones. Frequency rate of ctenoid scale on trunk 71.4% in count of 21 scales. Shape rather broad, circular on head ; hexagonal, more or less narrowed anteriorly with linear lateral sides on trunk and tail. Caudal scales biassed in shape asymmetrically as seen in *M. abei*. Size 0.58×0.52 mm on head, 1.03×1.00 mm and 1.51×1.31 mm on trunk (cycloid and ctenoid scales respectively), $1.40–1.77 \times 1.38–1.53$ ($1.52 \times 1,42$) mm on tail.

Circuli set more or less equally on ctenoid scales, but they are sparser in distribution or disappear on posterior region of cycloid scale. Radii 10 on head, 14–15 on trunk, 18 on tail in number. Spines 34 in number on trunk, 30 on tail.

According to Jordan and Snyder's description of *T. ioturus* [$=T.$ *trigonocephalus*][8], this fish was known to have only ctenoid scales. But the specimen examined now is furnished with cycloid scales exclusively on the head, and with both cycloid and ctenoid ones mingled simultaneously on the trunk.

8. Jordan, D. S. and Snyder, J. O., 1900. A list of fishes collected in Japan by Keinosuke Otaki, and by the United States Steamer Albatross, with descriptions of fourteen new species. Proc. U. S. Nat. Mus., 23 (1213), 335–380, pls. 12.

20. *Oligolepis acutipennis* (CUVIER et VALENCIENNES)
(Pl. XI, J ; Pl. XII, L ; Pl. XV, K, L.)

Scale large, hexagonal, ctenoid on occiput, trunk and tail, except for minute cycloid scales which appear on occiput in proportion of 20% ; well broad, 1.23–1.27 times as wide as long, and this ratio is rather smaller on caudal scales. Size measures 1.37×1.08 mm on occiput (in ctenoid scale), 2.02×1.58 mm on trunk, and 2.56×2.07 mm on tail of the specimen of 58.7 mm long.

Circuli set rather thickly in distribution. Radii and spines largest in number on caudal scales, the former 14 on head, 16 on trunk, 23 on tail, the latter 17, 35 and 44 in number on head, trunk and tail respectively.

The scales of the present species closely resemble those of *R. gymnauchen* in general appearence, but differ from them by possession of developed posterior angle.

Dealing with this fish, DAY[9] says that scales are ctenoid, and that "none [of scales] in front of first dorsal fin." The present investigation results against DAY's description as follows : cycloid scales present besides ctenoid ones even on the occipital region. But it is in consensus with DAY's description that the scales are "angular."

21. *Eviota abax abax* (JORDAN et SNYDER)
(Pl. XII, A, B.)

Scale rather deciduous, ctenoid on both trunk and tail, widely hexagonal. Proportion of width to length 1.14–1.34 (average 1.25). Size large, measures 1.53–2.13×1.19–1.83 (1.66×1.32) mm on trunk, 1.60–1.94×1.31–1.68 (1.83×1.45) mm on tail, in the 41.7 mm long specimen ; 1.51–1.65×1.27–1.36 (1.60×1.29) mm and 1.76–2.00×1.40– 1.54 (1.87×1.47) on trunk and tail respectively in the other specimen of 44.0 mm long ; generally larger on tail than on trunk.

Circuli well developed, but rather sparse in distribution, given more thickly on lateral regions than on anterior region. Resting zone very vague. Both radii and spines larger in number, the former 23 on trunk, 19 on tail ; the latter 60 and 54 on trunk and tail respectively.

The scales of this species resemble in shape those of *O. acutipennis*.

By the present investigation it is again corroborated that the range of the non-scale zone of the head tallies well with that given by JORDAN and SNYDER[7] (p. 41), who point out that the zone extends to dorsal insertion and "backward a short distance along the dorsal base."

9. DAY, F., 1889. The Fauna of British India. Fishes-vol. 2, xiv+509, figs. 177.

EXPLANATION

Length in mm, which follows to specific name, total length of the specimens. The scales drawn on figures are picked up from the left side of the body, unless otherwiss stated.

PLATE X

Caudal scales

Type-A:
Fig. A. *V. hanae*, 96.3 mm. ×100.
Fig. B. *A. lacticella cosmurus*, 42.4 mm. ×50.
Fig. C. *A. lacticella lacticella*, 44.6 mm. ×50.
Fig. D. *C. gulosus*, 98.1 mm. ×60.
Type-B:
Fig. E. *P. elepoides elapoides*, 67.2 mm. ×25.
Type-C:
Fig. F. *P. zacalles*, 138.8 mm. ×25.
Fig. G. *C. dolichogunathus*, 64.5 mm. ×50.
Type-D:
Fig. H. *P. elapoides zonoleucus*, 42.8 mm. ×35.
Fig. I. *Z. farcimen*, 32.6 mm. ×45.
Fig. J. *S. geneionema*, 90.5 mm. ×25.
Fig. K. *A. ornatus campbelli*, 94.7 mm. Outline. ×12.
Figs. L and M. *R. giurinus*, 79.3 mm. Outline (L, ×15) and detail of a part (M, ×35). Showing the trace of the disklet denoted in the text.

PLATE XI

Caudal scales (continued)

Type-D (continued):
Figs. A. and B. *B. fuscus*, 48.2 mm (A, ×15), and 62.5 mm (B, ×13). Outline, spines omitted.
Fig. C. *T. obscurus obscurus*, 88.3 mm. ×15.
Fig. D. *A. lactipes*, 44.0 mm. ×35.
Fig. E. *R. gymnauchen*, 76.2 mm. ×25.
Fig. F and G. *A. flavimanus*, 122.9 mm. Outline (F, ×15) and detail of a part (G, ×35).
Fig. H. *M. abei*, 32.2 mm. ×35. Dorsal part one.
Fig. I. *T. trigonocephalus*, 61.5 mm. ×30.
Fig. J. *O. acutipennis*, 58.7 mm. ×25.

PLATE XII

Caudal and abdominal scales

Caudal scales, Type-D (Continued):
Fig. A. *E. abax abax*, 41.7 mm. Part of the scale. ×25.
Abdominal scales:
Fig. B. *E. abav abax*, 41.7 mm. ×25.
Fig. C. *V. hanae*, 96.3 mm. ×150.
Fig. D and E. *A. lacticella lacticella*, 44.6 mm. Inside base of pectoral (D) and the region above pectoral (E). ×100.
Fig. F. *Z. farcimen*, 37.1 mm. ×45.
Fig. G. *B. fuscus*, 48.2 mm. ×35.
Fig. H. *R. gymnauchen*, 76.2 mm. ×25.
Fig. I. *R. giurinus*, 62.0 mm. ×15.
Figs. J and K. *M. abei*, 32.2 mm. Cycloid (J) and ctenoid (K). ×50.
Fig. L. *O. acutipennis*, 58.7 mm. ×25.
Fig. M. *A. lactipes*, 44.0 mm. ×35.

PLATE XIII

Abdominal scales (continued)

Figs. A-D. *A. ornatus campbelli*, 94.7 mm. Outline (A, ×12), and details of parts (B-D, ×50), which show the discontinuities of the circulus arrangement: the lst-3rd rings of the scale in question.
Figs. E and F. *A. flavimanus*, 122.9 mm. Outline (E, ×15) and detail (F, ×35).
Figs. G and H. *P. elapoides zonoleucus*, 42.8 mm. Cycloid (G), and ctenoid (H). ×35.
Figs. I and J. *P. elapoides elapoides*, 67.3 mm. Regions above pectoral (I, ×25) and under pectoral (J, ×35).
Figs. K and L. *C. gulosus*, 98.1 mm. Outline (K, ×25) and detail of a part (L, ×50)

PLATE XIV

Abdominal and abnormal scales.

Abdominal scales (Continued):
Figs. A and B. *P. zacalles*, 138.8 mm. Regions above pectoral (A) and under depressed pectoral (B). ×35.
Fig. C. *C. dolichogunathus*, 64.5 mm. ×50.
Fig. D. *S. geneionema*. 90.5 mm. ×25.
Figs. F and G. *T. obscurus obscurus*, 88.3 mm. Cycloid (E, ×25); ctenoid, on the anterior (F, ×25) and the posterior (G, ×18) parts of the abdominal division.
Figs. H and I. *T. trigonocephalus*, 61.5 mm. Cycloid (H) and ctenoid (I). ×30.

Abnormal scales:
Fig. J. *Z. farcimen*, 37.1 mm. Caudal. ×25.
Fig. K. *R. giurinus*, 79.3 mm. Caudal. ×25.
Figs. L and M. *T. obscurus obscurus*, 88.3 mm. Intermediate (L) and abnormal (M). Posterior part of the scale. ×35.

PLATE XV

Cephalic scales

Figures drawn from nuchal region, unless otherwise stated.

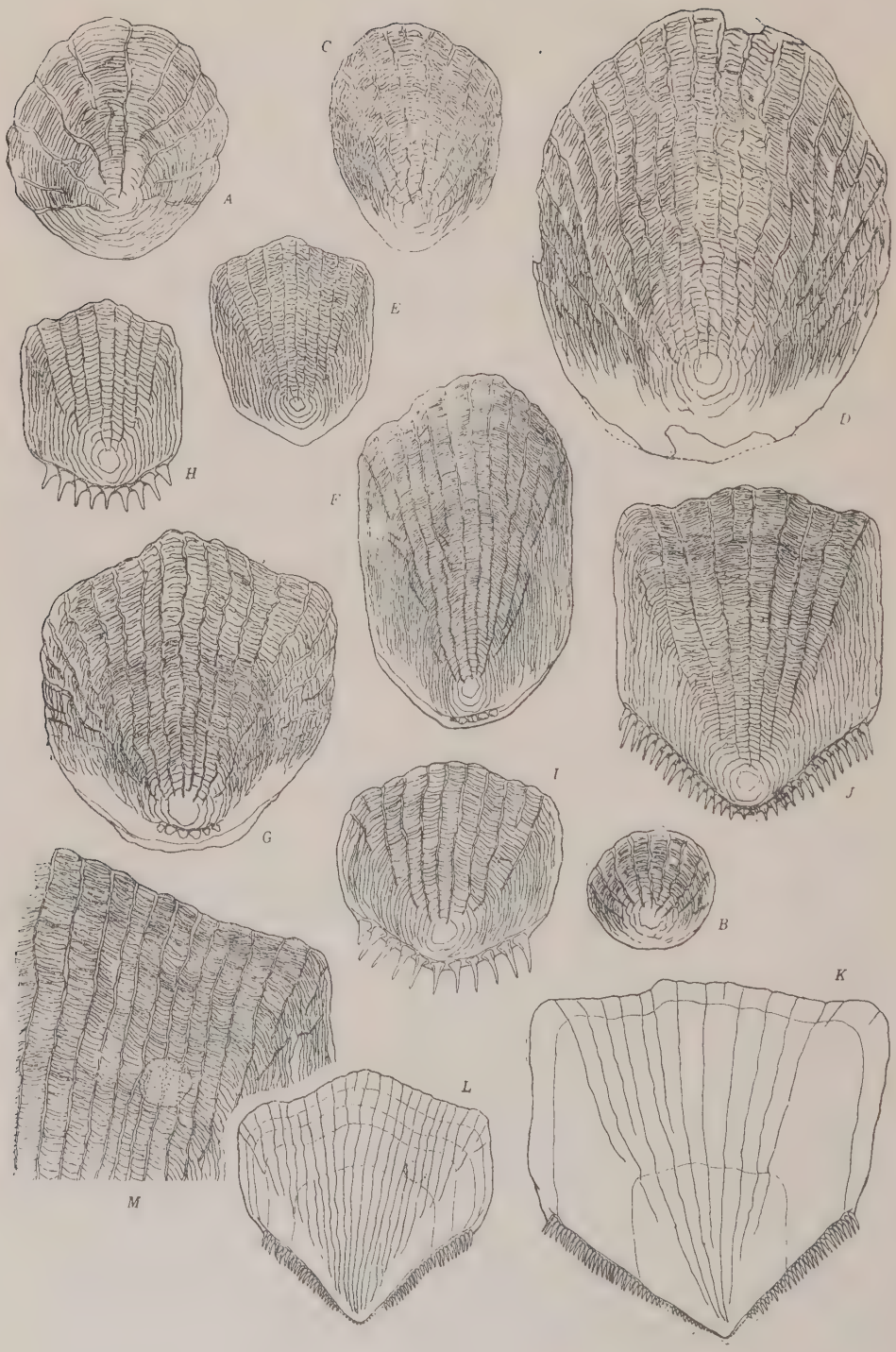
Fig. A. *B. fuscus*, 62.5 mm. ×35.
Figs. B and C. *R. giurinus*. Cycloid (B, 79.3 mm, ×50) and ctenoid (C, 62.0 mm, ×15).
Fig. D. *M. abei*, 32.2 mm. Upper part of operculum. ×50.
Figs. E-J. *A. ornatus campbelli*, 94.7 mm.
Figs. E-G. Outline (E, ×13) and detailes: antero-lateral (F, ×35) and posterior (G, ×25) parts, of cycloid scale.
Figs. H-J. Outline (H, ×13) rne details: antero-lateral (I, ×35) and posterior (J, ×25) parts, of ctenoid scale.
Figs. K and L. *O. acutipennis*, 58.7 mm. Cycloid (K, ×50) and ctenoid (L. ×25)
Fig. M. *A. lactipes*, 44.0 mm. ×35.
Figs. N and O. *A. flavimanus*, 122.9 mm. ×35. Cheek (N) and upper part of operculnm (O).

PLATE XVI

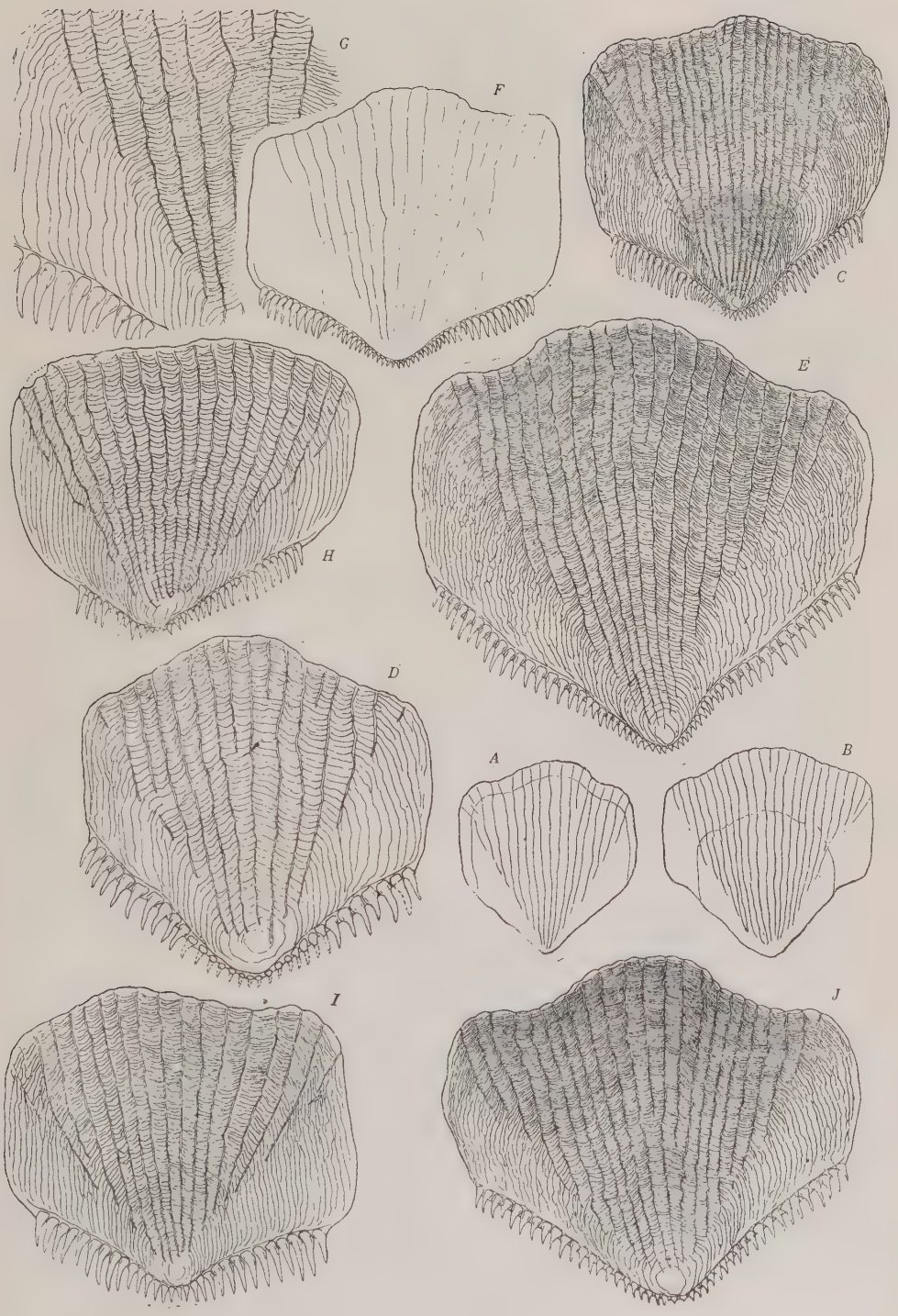
Cephalic scales (continued)

Figs. A-D. *A. flavimanus*, 122.9 mm. (continued). Nuchal region (A, ×25,) pentagonal occipital (B, ×30), and broad-bean shaped occipital: outline (C, ×15) and detail of spinous part (D, ×50).
Fig. E. *P. elapoides zonoleucus*, 42.8 mm. ×35.
Fig. F. *P. elapoides elapoides*, 67.2 mm. ×25.
Fig. G. *C. dolichogunathus*, 64.5 mm. ×50.
Figs. H. and. I. *P. zacalles*, 138.8 mm. Right side. Praeopercle (H) and occipital (I) regions. ×50.
Fig. J. *T. trigonocephalus*, 61.5 mm. ×35.
Fig. K. *C. gulosus*, 98.1 mm. ×60.
Figs. L-O. *S. geneionema*, 90.5 mm. Cheek (L, ×35), upper part of operculum (M, cycloid, and N, ctenoid, ×35) and occipital region (O, ×30).
Figs. P and Q. *T. obscurus obscurs*, 88.3 mm. Upper part of operculum (P) and nuchal region (Q). ×40.

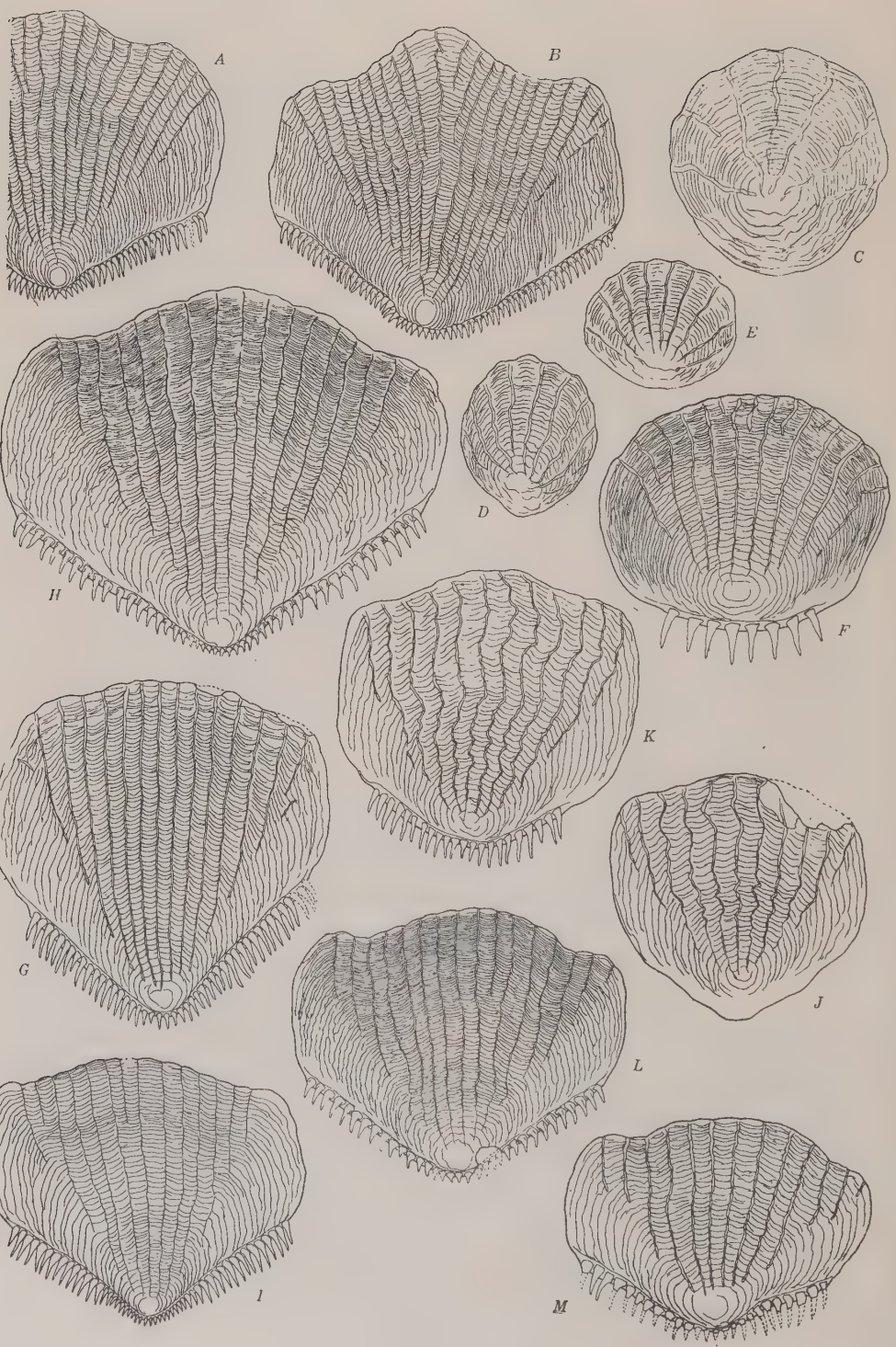
PL. X



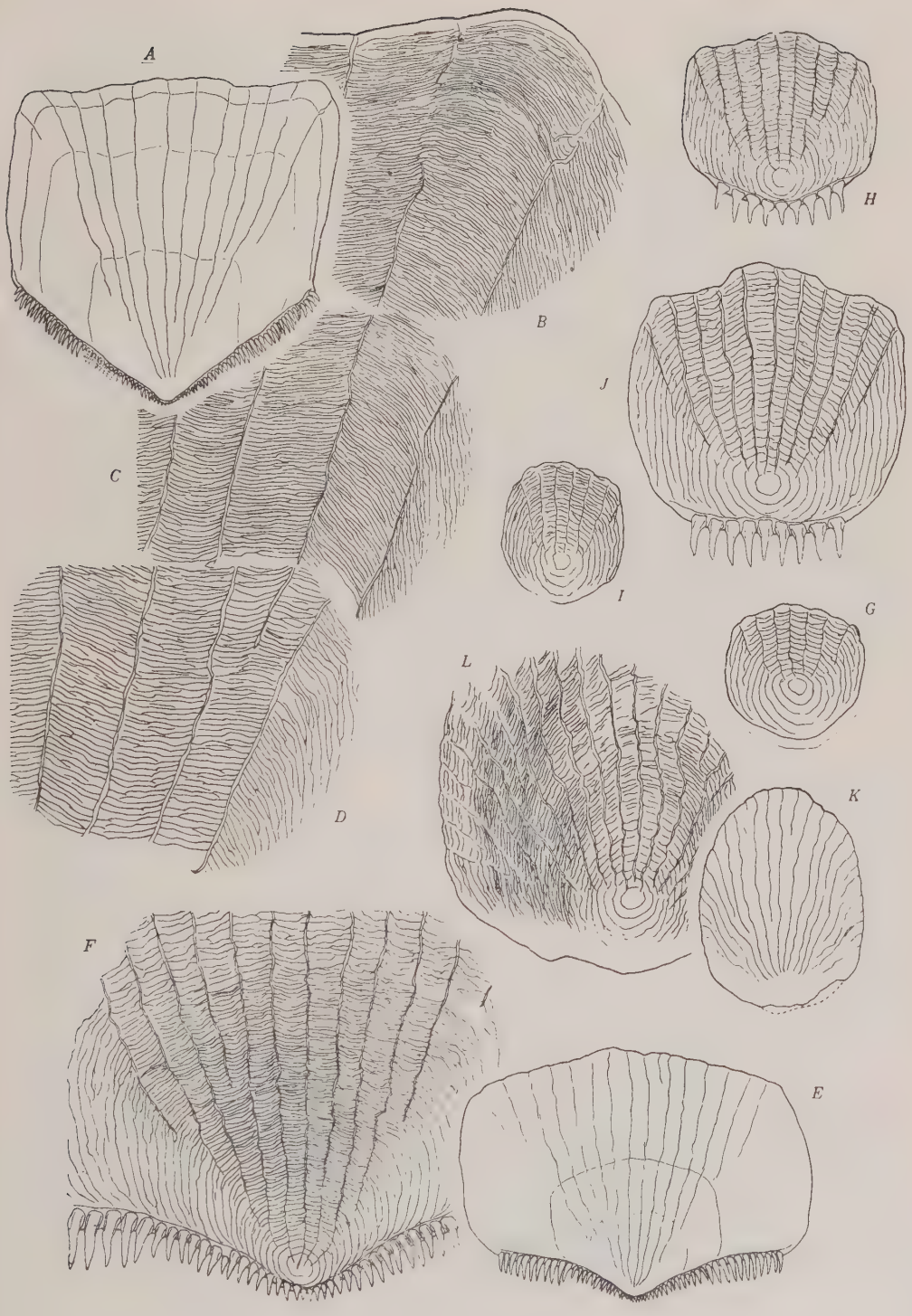
PL. XI



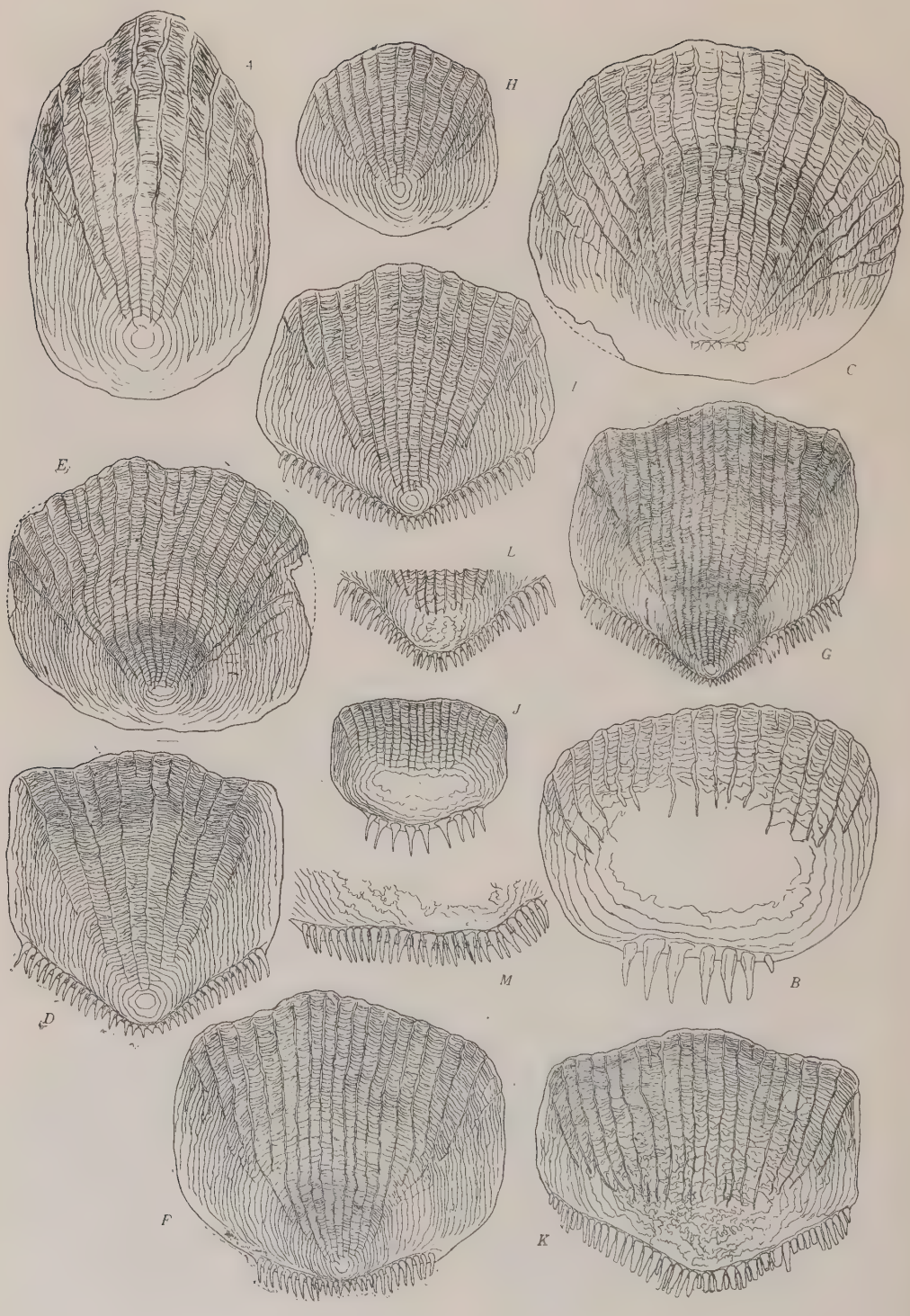
PL. XII



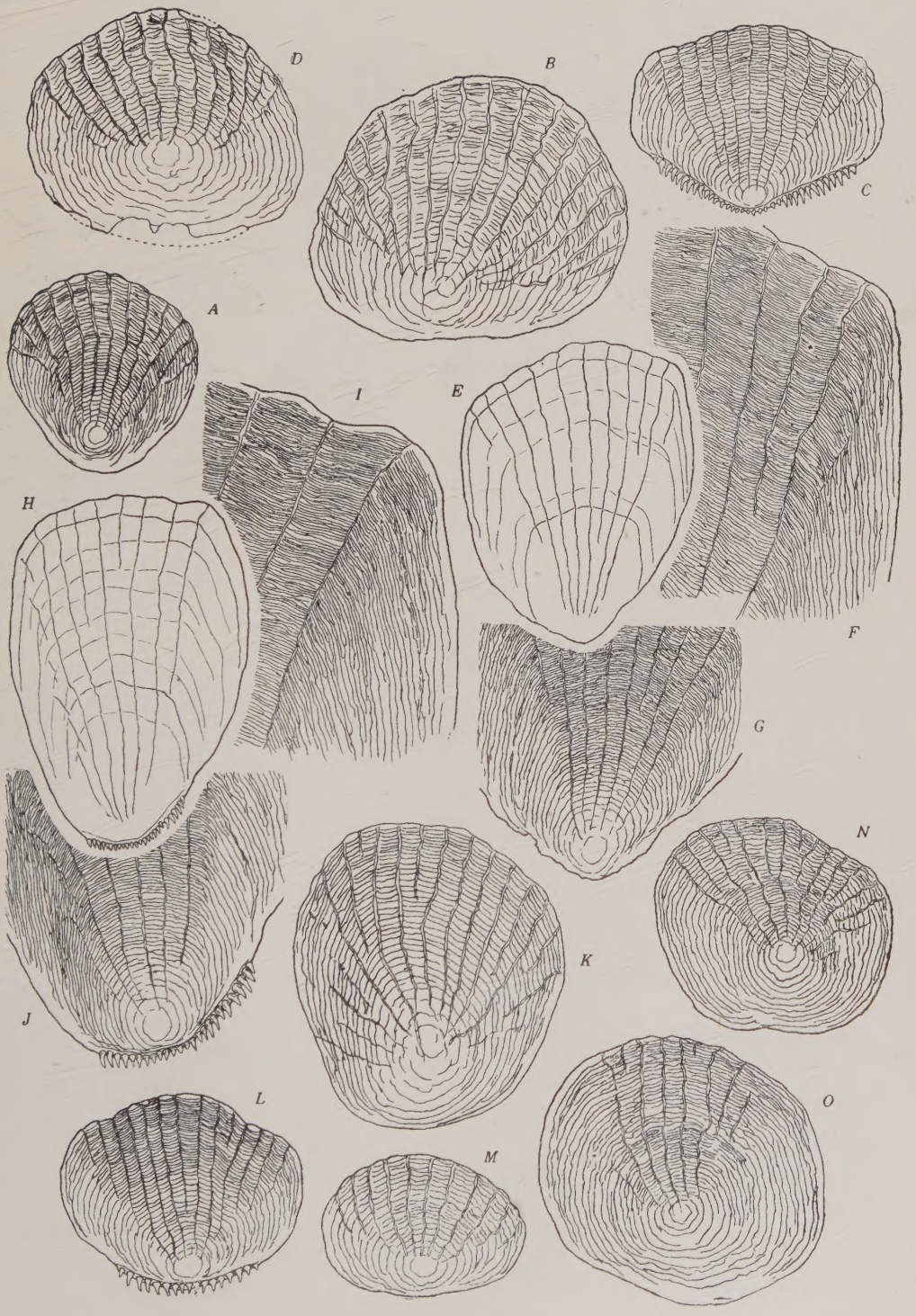
PL. XIII



PL. VIX



PL. XV



PL. XVI